The effects of early visual experience on cerebral RNA metabolism in the young chick.

Thesis

THE EFFECTS OF EARLY VISUAL EXPERIENCE ON CEREBRAL RNA METABOLISM IN THE YOUNG CHICK.

by

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C O N T E N T S

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ABSTRACT

1. A method for preparing nuclei in high yield from brain tissue of one-day old chicks was developed. These nuclei appeared to be essentially free from contamination by cytoplasm and endothelial cells. A further fractionation of nuclei according to size (i.e. of presumed neuronal and glial origins) proved impractical.

2. The activity and some characteristics of the enzyme RNA polymerase in the nuclei were determined and compared to the data available on the same enzyme from other sources. The activity of the chick enzyme was higher than that from mammals but was basically similar in its response to ions in the assay medium.

3. The activity of RNA polymerase was measured in three brain regions of chicks either exposed for 30 minutes to an imprinting stimulus or kept in darkness. There was a 34% higher activity in the forebrain roof of the exposed than the dark-maintained birds.

4. A new set of equipment for exposing chicks to the imprinting stimulus or diffuse light was built and tested, both by the birds' behaviour after exposure and by their relative degrees of incorporation of ^{14}C-uracil into brain RNA. These results were compared to those obtained previously on other equipment and found to be in general agreement.

5. Using the new eqipment, the effects of different periods of exposure to the stimulus on the birds' cerebral RNA polymerase activity were determined. These results were compared with those from the uracil incorporation experiments.

6. Possible extensions of the work are discussed in conjunction with other suggested directions in which such research could be pursued.

CHAPTER I

The characterisation of the mechanisms which underlie the behaviour of an organism is an awe-inspiring task, but one with which Man has been concerned since his earliest recorded days. From the concepts of visceral 'humour' to the recognition of the importance of the brain, and thence from phrenology to modern neurobiology, the problem of understanding how overt behaviour is produced and regulated, how experience is stored, modified, recalled and sometimes forgotten, appears more difficult the closer to the answer we get.

Several theories on the mode of action of the central nervous system have been presented (HEBB, 1949; KATZ and HALSTEAD, 1950; LASHLEY, 1950; HORN, 1967; MARR, 1970) since about 1950. They are all based upon the characteristic property of nerve cells (neurons) that they can directly, and specifically affect each other's activity, either positively or negatively, through synaptic connections. Some, but not all, the models of neural activity propose modification of the synapse as the mechanism of information storage. Those which do are theoretically testable by biochemical means, but those which do not, and depend for example, upon the frequency and temporal relationships of converging trains of nerve impulses upon the neuron to determine its response, clearly cannot be explored in this way. Obviously, in the study presented here, and in those similar ones to be discussed now, the former rather than the latter type of model is being tested.

In the simplest terms, stimuli impingeing on the organism are recorded by receptors in the sense organs (eye, ear, nose)

or skin and information about their characteristics (e.g. spatial distribution, intensity) are relayed to the brain via the relevant nerves as impulses. Actions are produced by the reverse process; signals from the brain travelling along nerve fibres to the muscles where they effect specific responses, seen grossly as behaviour. Between the input (afferent) and output (efferent) nerves is the complexity of the interneurons, nerve cells which do not directly connect with either receptors or effectors but only with each other and the terminals of the afferent and efferent nerves. It is probably mostly through modifications in the interconnectivity of these cells that information storage and integration occurs. The likelihood of a given input producing a particular response will be dependent on both the amount and type of interneuronal connections. The presence in a particular pathway of very efficient synapses (that is capable of causing the post-synaptic cells to 'fire' or not to fire every time impulses arrive at the synapse) will generate a high likelihood of the response occurring. Thus in this grossly simplified situation, the modification of synapses as a result of a specific input will relate a behavioural response to a stimulus in such a way that on re-presenting the stimulus the response will be evoked (all else remaining equal). In this sense there has been 'learning', at least as it applies to simple animal task or skill acquisition.

These synaptic modifications need not be of only one type; new synapses could be formed (synaptogenesis) or previous ones lost, or else differing degrees of efficiency could be achieved (facilitation or inhibition). The biochemical mechanism underlying these events is not yet resolved, nor indeed need there be

only one kind. Different synapses may be modified in different ways. The most likely means are:

a) changed amounts or activities of the enzymes responsible for neurotransmitter metabolism.

b) changed amounts or sensitivities of the neurotransmitter receptors.

c) changes in the efficiency of neurotransmitter release.

d) changes in the distance between the pre- and post-synaptic membranes.

e) increase in the size (i.e. 'contact area') of the synapse.

f) changed characteristics of the response of the post-synaptic cell in its conversion of the chemical message (transmitter) into an action potential.

All of the possible mechanisms for modifying the action of the synapse involve proteins, most of them probably alterations in protein synthesis or breakdown.

The durability of memory (that is, the so-called long-term memory) makes it likely that permanent changes in the structure of brain cells are required to encode it, and this has focussed research attention on to protein metabolism, for proteins, with their long half-lives (i.e. stability), known specificity and involvement with most cellular processes appear as the most likely candidates for effecting cellular modifications. (Polysaccharides and lipids could also be involved, but very little attention has been paid to them in this respect.) Further weight has been added to this view by studies on memory formation and recall in animals injected with drugs which inhibit RNA and protein synthesis. These substances seem to prevent the storage of long-term memories but do not affect short-term recall (see for example COHEN and

BARONDES, 1968; SQUIRE et al, 1973; AGRANOFF, 1965, 1967 and UPHOUSE et.al, 1974 for review). However their mode of action is less clear-cut than was originally thought because subsequent injections of saline or electrical stimulation can sometimes reverse the apparent memory loss (BARONDES and COHEN, 1968; FLEXNER and FLEXNER, 1967 and 1970).

The initial objectives of neurochemical research into behaviour phenomena have been, and are, to define the qualitative and quantitative changes which occur in metabolism, particularly that of the nucleic acids and proteins, in response to environmental stimulation and information storage. There is a large literature available on the experiments of this type, but only a few will be discussed in detail here to exemplify some of the problems which can arise in both their design and execution, and in the interpretation of their results.

Experiments with gross experiential effects (e.g. 'impoverished' versus 'enriched' environments; first exposure to light) have shown that large biochemical and structural differences can be produced in the brain, and particularly the cortex, of animals exposed to such treatments. The density and number of dendritic spines, in other words potential synaptic contacts, is decreased by visual deprivation or deafferentation (FIFKOVÁ, 1970a) and increased or modified by initial visual experience (FIFKOVÁ, 1970b; CRAGG, 1969a,b). Protein metabolism (measured as incorporation of radioactive amino acid) in retina, lateral geniculate nucleus and visual cortex is altered (increased) after a short period of first exposure of dark-reared rats to light (ROSE, 1967; RICHARDSON and ROSE, 1972), this apparently being localised

in neurons (ROSE et al, 1973). Rearing rats in environments which are enriched by 'toys' and handling results in increased size of the cortex and possibly of dendritic branching as compared to 'impoverished' environments (ROSENZWEIG et al, 1968, 1969). Results such as these indicated that changes of the sorts postulated to occur during learning did in fact exist; the goal of finding such changes after much smaller experiences such as task-learning has not yet been reached.

One of the major problems of trying to detect changes in metabolism during learning is that such changes, if they do exist, are likely to be very small, and so, in general, it is not possible to measure the increase or decrease of absolute amounts of substances in the tissue. As a consequence, the technique of injecting radioisotopically-labelled precursors of the substances has been used, the amounts of these incorporated being related to the rate of synthesis of the substance. For example to study RNA metabolism, radioactive uridine, orotic acid or uracil can be injected, all of these being incorporated mostly into RNA (after the necessary biochemical conversions). Although very tiny quantities of the precursor have to be used, so as not to disturb the system by significant increases in the precursor levels, and hence only very small amounts of radioactivity are incorporated, the availability of very sensitive radiation monitoring equipment makes the measurement of the extent of incorporation very simple. There are, however, serious drawbacks to this method, particularly for use in trying to measure net synthesis of macromolecules, viz., the rate of incorporation of the precursor into the macromolecule is affected by many other parameters than just that of synthesis (OJA, 1973). Figure 1 shows the interrelationships between precursor levels in

various metabolic compartments after an intraperitoneal injection (derived from that of OJA, 1967). Because of the difficulty of measuring the various input and output parameters of the compartments, it is generally assumed that the only factors influencing the incorporation rate of the precursor into brain macromolecules are those shown in Fig 2 (again from OJA, 1967). For this reason the only usual measurements made are of free and incorporated radioactivity in the brain and either in blood or liver. If the effects of environmental stimulation were partly to cause changes in the rates of metabolism (that is conversion of the precursor to other substances than the macromolecule in question) this would seriously affect the validity of the results. When possible, to minimize this effect, a precursor is chosen which can be shown, in the resting animal, not to be appreciably metabolised other than in the required way.

To further complicate the issue there are other factors present which make it difficult to relate the precursor incorporation measured to the rate of net synthesis:-

a) The specific radioactivity of the precursor pool in the compartment within which synthesis is occurring (and this may be a sub-cellular compartment, bearing little similarity to the whole cell or whole tissue with respect to the precursor) will directly affect the apparent rate of precursor incorporation. The specific activity is essentially a measure of the ratio of labelled to unlabelled precursor molecules in the pool and thus, even if the rate of synthesis and degradation of the macromolecule remains constant, if the precursor specific activity is higher in one animal than another there will be a higher rate of incor-

poration of radioactive precursor in the former than the latter. Thus, ideally, the specific activity of the precursor in its relevant pool ought to be measured in all such experiments; in practice this is often difficult to achieve.

b) Even when the pool specific activity remains constant, if both the synthesis and degradation rates increase there will be a higher 'throughput' of precursor into macromolecules, that is the proportion of new macromolecules synthesised after the introduction of the labelled precursor will be increased and hence a higher incorporation rate, which in this case does not equate with increased net synthesis. Indeed there may be a net degradation and higher incorporation.

c) A problem with the use of radioisotope-labelled biochemicals, which was only recognised recently, is the loss of tritium atoms from the precursor into water (and to a lesser extent other amino acids). After a 1hr ^{3}H-lysine pulse in the rat, 40% of the unincorporated radioactivity was present as $^{3}H_2O$ (TIPLADY, 1972) and 67% was as $^{3}H_2O$ from ^{3}H-leucine (SCHOTMAN et al, 1974). Similar effects were seen in the mouse (BANKER and COTMAN, 1971; DUNN et al, 1971). The use of ^{14}C-labelled precursors are in general more reliable for many studies, although their specific activity is lower, and their cost higher.

In many of the experiments to be discussed these problems will be taken into account when interpreting the results obtained.

Almost as many different learning situations have been used for neurochemical studies as there have been groups studying them. Hydén et al have examined the changes in both RNA and protein metabolism in response to learning situations. In a task where rats have to learn to use their non-preferred paw to reach

for food down a narrow tube, there were increases in RNA content in the neurones (hand-dissected) of the cortex of the hemisphere contralateral to the paw used. Similar changes were also found in Deiters cells (involved in balance control) when rats had to learn to climb a 45^{o} wire to reach food. When the animals learning paw transfer were still learning the task there was an increase in DNA-like RNA (speculated to be messenger RNA) whereas once they had learned the task it was of ribosomal type (by base ratio) (HYDÉN and EGYHAZI, 1962, 1963, 1964; HYDÉN and LANGE, 1965). Base ratio changes were also found in glia. Using a swimming task in goldfish in which they had to learn to compensate for a polystyrene 'float', SHASHOUA (1968, 1970) found very similar results, although his task was physically more strenuous in that the fish could not control their own rate of training. Both of these studies were measurements of absolute amounts of RNA and its composition and did not depend upon labelled precursor incorporation. They are therefore free from some problems of interpretation, those related to the biochemical analysis, but not those related to the specificity of the effects with respect to learning. Hydén's choice of brain region (sensori-motor cortex) has been criticised on the grounds that it is necessary for control of the forelimb, and hence side-effects due to novel motor activity cannot be ruled out (see BATESON, 1970). (These problems will be discussed for all the various types of experiments later in this chapter.)

In a later series of experiments Hydén et al examined the response of protein metabolism to the acquisition of transfer of handedness. By injecting ^{3}H-leucine into rats trained for

4, 6 or 8 days they found that there were differences between the trained and control animals in both the degree of incorporation into total protein and its anatomical distribution. After 4 days' training there was a high trained/control incorporation ratio in hippocampus, dentate nucleus and mammillary bodies, and a low ratio in cortex, reticular formation and septum; after 6 days this was essentially reversed and after 8 days the only difference was a high ratio in the thalamus. They also found that the trained animals always had lower incorporation than the controls (HYDÉN and LANGE, 1972). This is suggestive of changes in the whole body conditions affecting the distribution of radioactivity or its specific activity.

In addition to these changes in ^{3}H-leucine incorporation, there was an increase in the amount of S100 protein (a brain-specific protein well characterised by gel electrophoresis) in nuclei from neurons in the hippocampus. This was later found to be due to the presence of two forms of the molecule, one with a higher calcium content than the other. The presence of anti-sera to S100 impaired the ability of the animals to learn the task (HYDÉN and LANGE, 1970). The high-calcium form of S100 may have been produced as a result of a specific calcium increase in the brains of trained animals; both before and after the learning of the task there was only low-calcium S-100 present in the neuronal nuclei (HALJAMÄE and LANGE, 1972). Whether these changes in protein metabolism are directly related to the task-learning process, and hence to memory storage has not been shown. The effects of S-100 antisera could have been due to disruption of necessary basic neuronal processes, and not of those specifically involved with the learning of the particular task.

The work of Glassman and his colleagues has been focussed on the changes which occur in RNA metabolism in the mouse when it is trained to avoid an electric shock through its feet by jumping to safety on to a ledge. The mouse was warned about the impending shock by a light and buzzer. It was compared with two types of 'control' animals, one which was in a cage away from the 'jump-box' and another, a 'yoked' control, which received identical light, buzzer and shocks to the trained animal but could not escape them. The RNA metabolism was measured by incorporation of uridine, the trained animal receiving either ^{14}C- or ^{3}H-uridine and the yoked control the other isotopic form. In almost all their experiments this group have used intracranial injections to deliver the precursor. This is questionable on two grounds: the first is that this injection route requires anaesthesia and is probably more likely to interfere with subsequent performance than, say, a subcutaneous or intraperitoneal route. The second is that the distribution of the precursor throughout the brain may not follow that supplied in the blood and so localised specific activity changes are more likely to occur. It does have the advantage of producing higher radioactivity within the brain because it avoids both loss into other tissues and 'blood/brain barrier' effects.

In trained mice there was enhanced incorporation of uridine into RNA in the diencephalon, a result confirmed by autoradiography, but which lasted less than 30 minutes after training. A similar increase was found in the radioactivity in the polysome fraction of the trained animals, with the effect being still present 60 minutes after training (ZEMP et al, 1966 and 1967;

KAHAN et al, 1970; ADAIR et al, 1968a). In a classical conditioning experiment no such differences in RNA metabolism were found (ADAIR et al, 1968b), the discrepancy being explained as due to over-training in the earlier jump-box experiments. However this group also claimed that while there was elevated uridine incorporation into trained mice there was no difference between yoked controls, quiet controls and random-shocked controls, thus apparently showing that the light/buzzer/shock stimuli had no effect on the brain RNA metabolism. This analysis was shown to be incorrect by BATESON (1970), namely that there was a difference between the ratio of trained : quiet and trained : yoke. Although the former was greater than the latter there was a significant increase in incorporation in the yoked controls as a result of their treatment. They have since measured uridine incorporation during extinction of the learned jump-box behaviour and found a similar increase in the trained animals versus naive controls. Because the stress is assumed to be less in the extinction experiment than in the learning one they have proposed that the changes found in RNA metabolism were due essentially to learning (COLEMAN et al, 1971). In the light of their later experiments in which increases in the phosphorylation of nuclear proteins was found after a 'reminding' exercise (that is the previously-trained animal was placed in the experimental situation but not given any stimuli), this interpretation is still open to question (MACHLUS et al, 1974). As a measure of stress in such experiments as these it would be interesting to know if there are changes in corticosteroid levels, which are increased by stress and can influence the rate of protein and RNA metabolism (JAKOUBEK et al, 1970, 1972), although COLEMAN et al (1972) have shown that the absence of adrenal and

pituitary hormones are not necessary for the increased incorporation of uridine in rats. An interesting sex difference was found in mice in that the normal females learned the task but showed no clear increase in incorporation, whereas the ovariectomised females responded exactly like males.

To avoid some of the behavioural and biochemical problems inherent in mammalian learning studies some groups have used the simpler invertebrate systems, for example headless cockroach, snail and Aplysia. Headless cockroach preparations can be trained to keep their legs above a saline bath by passing an electric current through the bath so that each time the leg is lowered it receives a shock (HORRIDGE, 1962). A control can be yoked to the trained insect so that both receive the same number of shocks. Using this system KERKUT et al (1970) showed that there was increased incorporation of precursors into RNA and protein in the abdominal ganglion. They have also found decreases in the specific activity of acetylcholinesterase and glutamate decarboxylase in the metathoracic ganglia of the trained insect (OLIVER et al, 1971). There does not appear to be a close relationship however between the incorporation studies and the enzyme activities. In the snail, which was taught to keep its tentacles retracted, there was increased incorporation of uridine into RNA and leucine into protein in the brain of the trained animal as compared to its yoked and quiet control, and in the yoked control than in the quiet control. There also appeared to be selective high incorporation into certain protein fractions, as produced by gel electrophoresis, in the trained animals (EMSON et al, 1971). In contrast to the cockroach, occurring over approximately the same time period

there was an increase in the activity of acetylcholinesterase, in both trained (60%) and yoked control (40%). The disappearance of this increased activity parallelled the loss of the learned behaviour. These experiments are useful because the neurones of snail brain have been partially characterised as to their responses (depolarisation or hyperpolarisation) to ACh and tentative conclusions can be drawn as to the relationship between the biochemical and behavioural events (KERKUT et al, 1973).

A category of experiments which differs radically from those discussed so far is involved with the search for specific 'memory molecules', that is substances which are capable of eliciting specific behaviour patterns and which are produced selectively as a result of an animal's exposure to a particular situation. The major proponent of such an approach (which has waned in favour sharply over the past few years) is Ungar. Rats were trained by electric shock to avoid the dark part of a cage and remain in the light part. Extracts from the brains of such trained animals were injected into untrained animals and their subsequent behaviour tested. The injected animals spent significantly more time in the light than the dark side of the cage, as compared with controls which were injected with an extract from an untrained rat. The active component of the extract was analysed and found to be a polypeptide of 15 amino acid residues. Synthesis of this substance (called by Ungar 'scotophobin') showed that it had the expected effects on behaviour. (UNGAR et al, 1968; UNGAR et al, 1972). Ungar sees the action of such substances as 'switches' which operate specific pathways through the CNS, hence using the known connectivity of the brain but deciding among the myriad

possible networks by chemical means. However several serious criticisms have been made, both of the biochemical aspects (STEWART, 1972) and of his behavioural techniques and measures (GOLDSTEIN, 1973). The latter is the most damaging because it points out that, irrespective of errors in the biochemical analysis, the measures of dark-avoidance are as well related to increased motor activity and that there is an inherent high degree of stress in the training procedure for which no control at all is used. This, coupled with a significant rate of failure to reproduce these results in other laboratories casts large doubts as to the validity of this result.

The problem of controls for non-specific (that is other than learning) effects in the training procedures in Ungar's work, raises the more general question of the possibility of compensating for such effects in any learning situation. The yoked control, as used by Glassman and Kerkut seems to be an excellent way of equalising stimulation and stress effects. However, as pointed out by BATESON (1970), the animals need repeated handling to return them to the floor of the jump-box, and although the yoked animals are also handled the relative degrees of stress in this may not be equal. Even in relatively stress-free situations, behaviour differences will automatically be produced between trained and yoked animals if the stimulus acts in a reinforcing fashion (CHURCH, 1964 and BATESON, 1970). Similarly, if in a training programme animals are trained to a criterion within a specified time and retested, rejection of animals which respond inadequately on retest may introduce systematic errors. The failure to respond may be due to low

attention or activity which are often not identifiable as such. Thus there would be no criterion for rejection of low-activity animals in the control group but the possibility of rejecting them in the trained group. Thus behavioural differences may be introduced into the results. If there were a correlation between motor activity and the biochemical measure then a difference between the groups in terms of biochemical effects would also be created (for such effects see, for example, TIPLADY, 1972).

A more rigorous control procedure is to use the trained animal as its own control. One side of the brain is trained and the other side is not, and the effects of the training compared within the one animal. This approach was used by Hyden (see earlier), and METZGER et al, (1967) and HORN et al (1973). Theoretically this process ought to equalise such effects as precursor supply and hormonal influences, but in practice it has been shown that differential blood flow can occur between the trained and untrained hemispheres (BONDY, 1974) which could result in an inequality in the distribution of small molecules. Hence the observed biochemical change will be due to a combination of specific and non-specific effects.

Viewed overall, the results of the large number of experiments into the biochemical changes which occur during or after an animal is trained to perform a novel task suggest that specific changes in macromolecular metabolism do occur more as a consequence of the training than non-specific side effects. However, as has been pointed out, all these experiments suffer from ambiguities, some of which are a result of the biochemical techniques and some of the training design. It may be that no adequate

control can be developed for such situations, and that the elements of arousal or stress are necessary preconditions for learning. Indeed the 'control' animals themselves are probably 'learning' to some degree, either by superstition (HERRNSTEIN, 1966) or by associating the characteristics of the treatment situation with noxious stimuli. It is probably only by the use of a variety of different training situations and controls that such problems can be circumvented.

So far there has been no discussion of the series of experiments performed by Rose, Bateson and Horn on the responses of brain metabolism of the young chick to imprinting stimuli. As these experiments relate very closely to those to be described in later chapters (III to VIII), they have been omitted and are described in some detail in the next chapter.

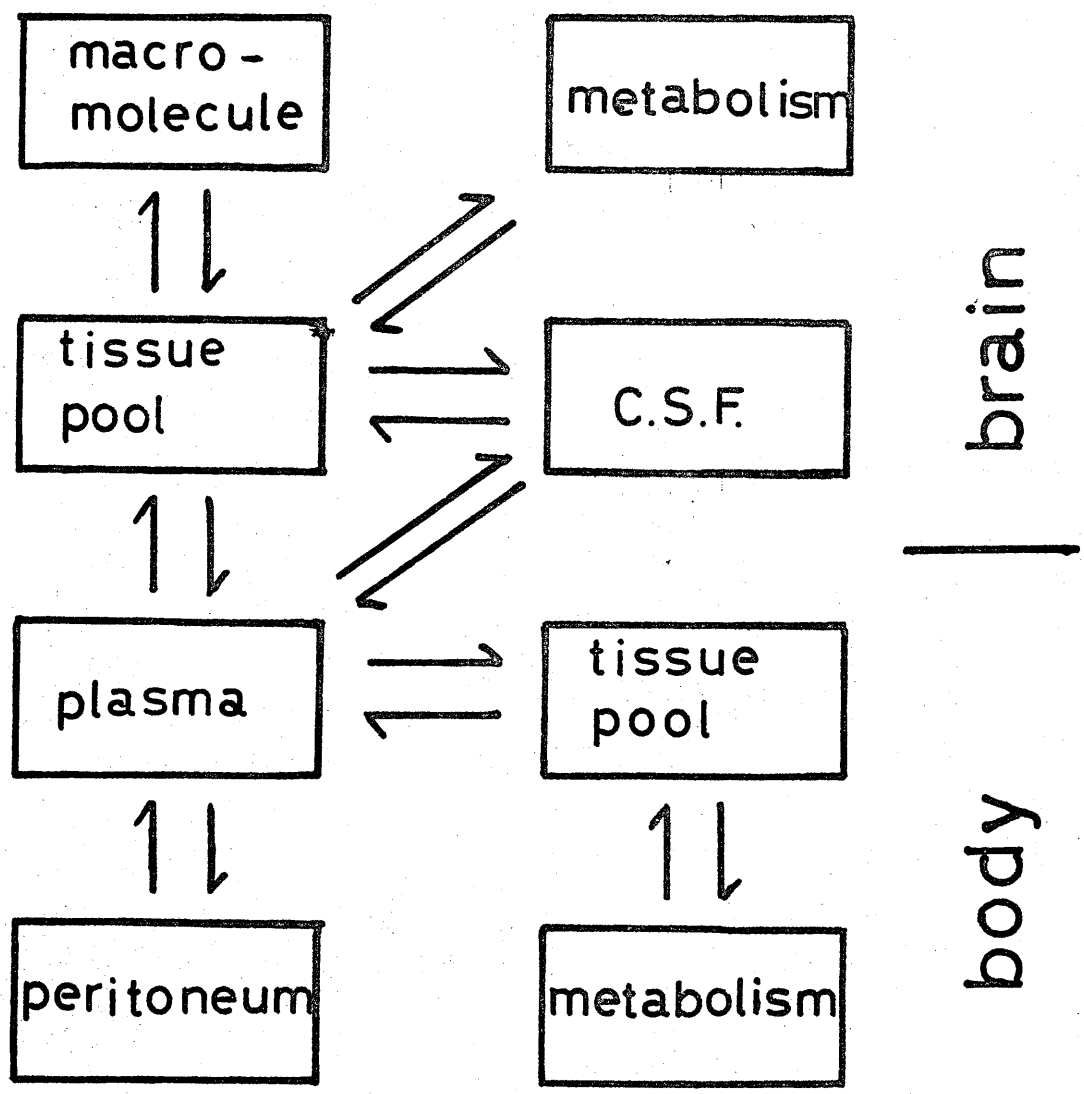


Fig. 1 Distribution of a radioactively-labelled precursor of brain macromolecules after injection into the peritoneum.

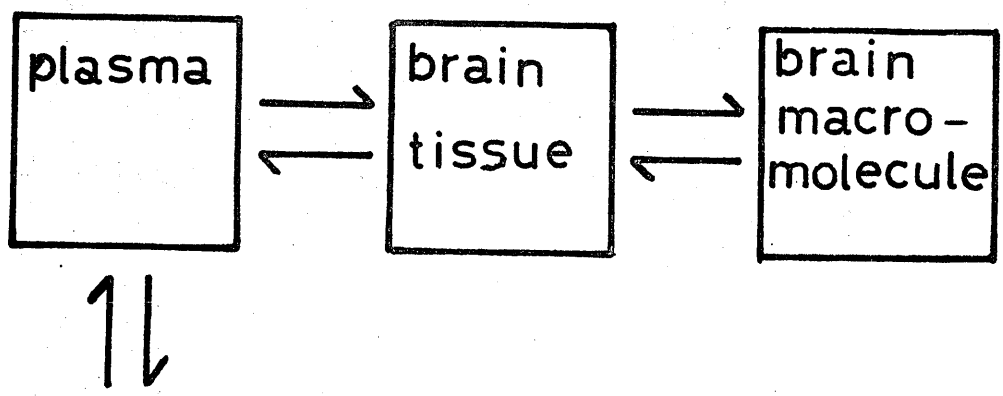


Fig. 2 Assumed metabolic compartments which influence the incorporation of a precursor into brain macromolecules.

CHAPTER II

One of the problems met with in most neurochemical studies of behaviour change is that the training situation is only one of a large number of learning situations which the animal has encountered (although in some cases, for most laboratory animals, they will certainly be the most stressful) and hence large alterations in brain metabolism are not expected. Indeed many of those found so far are surprisingly large. In an attempt to maximise such changes, Bateson, Rose and Horn have successfully performed experiments using young chicks in a situation in which they rapidly acquire a preference for a stimulus without the need for either overt reward or punishment. This type of learning in the young bird is called 'imprinting', and has been intensively studied for many years, although systematic laboratory experiments are relatively recent (for reviews see BATESON, 1966, 1972; SLUCKIN, 1972; SMITH, 1969).

When birds are exposed, within a few days of hatching, to a conspicuous visual stimulus, they begin to learn its characteristics and to limit their preferences to it. This is such that if they are removed from the object they will actively work to find or approach it and will avoid other objects even if they are relatively similar. This phenomenon was first studied in detail and defined by LORENZ (1935) although it had been recognised much earlier (SPALDING, 1873). Lorenz noted that such learning was most marked very early after hatching, and called the time over which imprinting occurred the 'critical period' because he thought that if no imprinting occurred during this

time, it could not subsequently occur. It was later shown to be less precise than this and has been renamed the 'sensitive period' (BATESON, 1966). In this respect it is similar to other periods of development when the propensity to learn new behaviour patterns is high (see THORPE, 1956 and 1961). The types of stimuli used have been very varied, including coloured cubes, flickering lights, rotating discs and model hens (LORENZ, 1935; FABRICIUS, 1951; HESS, 1959; JAMES, 1960; BATESON, 1964). Some appear to be better than others in eliciting imprinted behaviour, there being colour preferences for the red end of the visual spectrum (JAYNES, 1956; SCHAEFFER and HESS, 1959), for size (FABRICIUS and BOYD, 1954; SMITH and HOYES, 1961) and for the direction of movement (MOLTZ, 1963). These variations and discrepancies between them could be due to genetic differences, rearing conditions or the exposure situations (see for example, GRAVES and SIEGEL, 1974). In general, however, it would seem that the more conspicuous the stimulus to the human eye the better it will be as an imprinting stimulus.

The degree to which a bird has imprinted on a stimulus is best tested, as in any visual discrimination learning study, by presenting the bird with the choice between the imprinted stimulus and another equally effective object. A difficulty with such a test is that the bird's approach to the first-selected stimulus may be self-reinforcing because it takes it away from the non-chosen stimulus which may therefore become less effective. Thus it is preferable if the effectiveness of the chosen stimulus decreases as the bird approaches it. This can be done either by decreasing the conspicuousness of the stimulus on approach or by

causing the bird to recede further from the chosen stimulus the more it tries to reach it (see BATESON and WAINWRIGHT, 1972).

There appears to be an approximate correlation between the characteristics of imprinting and the type of bird in which it is observed, which may account for some of the variability in the results which have been obtained. In the wild state imprinting has obvious usefulness in keeping the young birds close to the protection of their mother. This is especially necessary for species in which the young can leave the nest within a few hours after hatching, e.g. Mallard (Anas platyrhynchos L.) and domestic chicks (Gallus domesticus). The majority of work on imprinting has been performed on such birds. Chicks seem to learn sufficiently detailed characteristics of objects around them that they can not only identify their mother but also individual siblings. This latter is evidenced by the development of 'pecking-orders'. There are also effects of imprinting on later sexual behaviour, but these are much more species-specific than the filial responses. In dimorphic species, where the adult male and females are visually distinct, imprinting in the male is much more likely to influence mate selection than in the female, whereas in monomorphic species both sexes are often affected in sexual behaviour by prior experience. In parasitic species, like the cuckoo, imprinting on the 'parents' is clearly of no relevance to mate selection. However several factors other than imprinting are probably involved in mate selection, such as specific hormone-induced behaviours (ANDREW, 1964) and learning around the time of sexual maturity (SIEGEL and SIEGEL, 1964). In general sexual behaviour seems to be influenced by imprinting only in so far as

it creates preferences rather than exclusive choices (SCHEIN, 1963) although there are notable exceptions (e.g. SCHUTZ, 1965).

The learning process which underlies imprinted behaviour has been the subject of much speculation and disagreement. Adopting the 'neuronal model' hypothesis of SOKOLOV (1960), SALZEN (1967) proposed that the orientation of chicks to the imprinted stimulus and avoidance of other objects resulted from their 'matching' of the characteristics of the imprinted and non-imprinted objects with a mental 'model'. Those objects which did not match would be avoided. However such a model does not account for the difference between orientation and the subsequent response which can be approach or fleeing. Nor does it explain the fact that birds will actively work for novel aspects of the stimulus, which will not match with the neuronal model.

BATESON (1971, 1972) has adopted an 'exposure' model in which the bird can initially respond to a range of stimuli, but that its preferences are increasingly restricted. Once selection of an appropriate stimulus is made it acts as a consummatory stimulus to prevent searching and initiate imprinting. In this respect it differs from classical associative learning where pairing of significant and insignificant stimuli is made (at least in the laboratory situation, in the wild state this may well not hold true), although as he points out, the absence of such pairing may only be due to our lack of identification of it. This approach has been criticised by DIMOND (1970) on the grounds that if the stimulus does act as a consummatory stimulus it should reinforce the preceding behaviour (i.e. searching) rather than the subsequent behaviour (i.e. imprinting). It is, however, only one example in the large number of learned behaviours which

occur with no obvious involvement of punishment or reward which in general have been little explored.

In a series of experiments, Bateson, Horn and Rose have investigated the biochemical responses in the chick brain during the acquisition of imprinted behaviour. The apparatus in which the chicks were exposed to the stimulus or to the 'control' environments and subsequently tested as shown in Figs. 17 and 18 (Chapter V). Briefly, all birds were reared in darkness until approximately 18 hours post-hatch. The stimulus-exposed (i.e. imprinted) birds were placed in individual pens so that they could see the stimulus (a flashing, orange light) but not approach it, and also overhead, diffuse, plain light. One group of control birds were placed in identical pens to the imprinted birds but could only see the diffuse light and another group were left in the dark brooder. After the period of treatment all the birds in each group were tested in an alley, and then killed. Their brains were dissected into three regions, forebrain roof, forebrain base and midbrain. (This is shown schematically in Fig 3.) The cerebellum and hindbrain were discarded and the midbrain separated from the forebrain by a cut just anterior to the posterior commissure. This portion therefore contained the intact optic tecta and the various nuclei of the mesencephalon. The forebrain was divided into an upper and lower portion (roof and base) by a cut which ran from the anterior tip to the dorsal posterior ridge. Thus the roof contained the hyperstriatum (including the Wulst) and most of the neostriatum, whereas the base contained the palaeostriatum and the thalamus.

The first study of changes in metabolism during the imprinting was done using ^{3}H-lysine as the precursor for protein

(BATESON et al, 1969, 1972). The birds were of two types, 'early-hatchers' and 'late hatchers', the former having hatched 6-9 hours before the midpoint of the hatch and the latter 6-9 hours after the midpoint. Consequently, although all the birds were the same age with respect to the hatch when exposed to their respective conditions they were not the same with respect to the onset of incubation. The order of the treatments is shown in Fig. 4. The stimulus-exposed and diffuse light-exposed birds were exposed to their treatments for 105 minutes and all groups incorporated the labelled precursor for 90 minutes, although these were not congruous, but overlapping. The lysine incorporation was higher in the forebrain roof of the stimulus-exposed (early-hatchers) birds than the same region in the dark-maintained birds (early-hatchers). There was no difference in any other region of the early-hatchers or in any brain region of the late-hatchers. The test of the behaviour of the birds showed that both the stimulus-exposed and diffuse light-exposed early-hatchers approached the stimulus faster than the dark-maintained birds whereas in the late-hatchers the stimulus-exposed birds approached faster than both the other groups. This difference in behaviour may have been related to the different developmental ages of the early- and late-hatchers.

However there are problems in the interpretation of these results. As discussed in Chapter I, the use of ^{3}H-labelled precursors prevents the acid-soluble radioactivity being taken to define the pool size, and so incorporation effects due to an increase in the specific radioactivity of the lysine cannot be ruled out. The length of the pulse (90 minutes) was rather long for this precursor because it is so rapidly incorporated into protein (HAYWOOD et al, 1973; HAMBLEY et al, 1974a), and the

longer the pulse the greater the loss of tritium and possible metabolism of the lysine. In spite of this, as a preliminary experiment it did indicate that there were detectable, specific changes in protein metabolism occurring during the development of imprinted behaviour, which did not occur on first exposure to plain light.

In the second study ^{3}H-uracil was used as the precursor for RNA because its rate of incorporation was apparently lower than that of lysine into protein, and so could be given in a longer pulse. By use of ^{14}C-uracil and ^{14}C-lysine, from which there is minimal loss of radioactivity, this has proven to be so (HAYWOOD - unpublished results) although the criteria used by Bateson et al at that time were not reliable guides. Their measure of the extent of incorporation was $\frac{\text{acid insoluble dpm/mg protein}}{\text{acid soluble dpm/mg protein}}$ (dpm ≡ disintegrations per minute). However the ratio $\frac{\text{dpm in lysine}}{\text{dpm in water}}$ may not have been, and probably was not, the same as the ratio $\frac{\text{dpm in uracil}}{\text{dpm in water}}$; in other words the rate of tritium loss was different in the two precursors. Thus the <u>true</u> extent of precursor incorporation into product would have been greater for uracil than for lysine if there had been a much higher loss of tritium from the former than the latter.

Bateson et al then performed two experiments using ^{3}H-uracil as precursor. In the first, the treatment time of all three groups (stimulus-exposed, diffuse light-exposed and dark-maintained) was 160 minutes with a pulse time of 150 minutes, this being within the treatment period (ROSE et al, 1970; BATESON et al, 1972). The incorporations in the forebrain roof and base of the stimulus- and diffuse light-exposed birds did not differ from each other, but both were higher than in the corresponding regions of the dark-

maintained birds. In the midbrain the incorporation was higher in the stimulus-exposed birds than in both the other groups. Thus a more widespread pattern of changes was found in uracil incorporation than that found for lysine. Bateson et al interpreted this as being due to non-specific visual stimulation, particularly in the midbrain which contains the optic tecta and which would be expected to respond to first exposure to light (see for example this treatment in the rat, ROSE, 1967). Consequently they examined the effects on uracil incorporation of two different times of exposure, both less than 150 minutes, and compared these results to those in birds which had been dark-maintained for the whole of the incorporation period (procedure shown schematically in Fig 5 and the acid-insoluble specific radioactivities in Fig 6). The uracil incorporation was higher in the forebrain roof of the stimulus-exposed birds than both the diffuse light-exposed and dark-maintained birds after 76 minutes of exposure, but no differences were found between any regions of any other types of birds at either time. This seems to fit in with the earlier hypothesis that an initial specific increase in incorporation due to specific stimulation was followed by a general increase due to non-specific stimulation. These two experiments are open to criticism of their experimental design. Firstly, the 150-minute exposure time was not repeated <u>in the same experiment</u>. It is possible, therefore, that the 150-minute results may not have been the same as found in the earlier experiment, especially as the behavioural and metabolic activities of the birds vary from hatch to hatch and season to season. (This may be due to incubation conditions and the genetic stock of the birds - see BATESON, 1974). Secondly, the birds against which the exposed groups were compared were not

given the same amount of handling, that is they were not transferred to the same room as the other birds for equivalent lengths of time. Thus there were no 38- and 76-minute dark-maintained birds, only 150-minute ones. If, as will be seen later for RNA polymerase, the dark-maintained birds were responsive to such transfer handling, the analysis of the results could have been altered, especially as all the results were related to the mean incorporation of the dark-maintained groups. The difference between the two exposed groups in the forebrain roof after 76 minutes is not affected by this problem, nor is the increase in incorporation between 38 and 76 minutes in the stimulus-exposed birds. Assuming that the two experiments were comparable, the sequence of changes in uracil incorporation was, a region-specific increase (roof) in the stimulus-exposed birds as compared to the other two groups by 76 minutes which was maintained up to 150 minutes, accompanied after 76 minutes by a sharp increase in the same region of the diffuse light-exposed birds. No changes occurred before 76 minutes in the other two regions, but after that time, and up to 160 minutes, there were rapid increases in the forebrain base of both exposed groups, and in the midbrain a very large increase in the stimulus-exposed birds.

The question of whether the elevation in uracil incorporation in the stimulus-exposed birds after 76 minutes was due to their acquisition of imprinted behaviour, and the later elevations due to non-specific visual stimulation, remained open. The simple exposure of 16-hour post-hatch, dark-reared birds to a few minutes of plain light has been shown to modify (i.e. enhance) their subsequent behaviour towards an imprinting stimulus (BATESON and WAINWRIGHT, 1972; BATESON and DIMOND, 1970;

SEABURNE-MAY, 1973), possibly by activating the previously little-used visual system. Hence the distinction between specific and non-specific stimulation is an arbitrary one, that is it is defined in terms of the information content of the stimulus within the context of the experimental design. The extent to which a bird exhibits imprinted bheaviour towards the stimulus is the criterion by which its degree of learning is assessed. The diffuse light-exposed birds have had no prior experience of the stimulus before testing, and consequently have had no opportunity to learn its characteristics; they therefore exhibit no preference for it. Thus, with respect to the stimulus, their treatment can be called non-specific stimulation. In fact, however, they are presumably learning other characteristics, namely those of the apparatus in which they are exposed, and so are essentially another category of 'learning animal', rather than a 'control'. Nevertheless, there is a qualitative difference between the two groups of birds, in that very dramatic and long-lasting changes in behaviour are elicited in the case of the stimulus-exposed but not diffuse light-exposed birds, viz. those of the imprinted animal.

The changes which were found in precursor incorporation could have been accounted for in several ways. Differential motor activity between the groups (which does occur to a certain extent during the exposure), stress or hormone levels are known to affect cerebral protein and RNA metabolism (JAKOUBEK, 1970 and 1972; TIPLADY, 1972). However, Bateson et al suggested that if the same bird could be used both as experimental and control such effects should be eliminated. Other more specific cellular changes such as altered rate of cell development, e.g. neuronal

or glial differentiation, general synaptogenesis, would probably not be eliminated, although such events could be intimately involved in the learning process in this instance.

Information transfer between the cerebral hemispheres has been observed in Peking ducklings in which only one eye was exposed to the imprinting stimulus (MOLTZ and STETTNER, 1962), and in chicks trained to avoid pecking objected coated in methyl anthranilate (CHERKIN, 1970). Bateson had also found that interhemispheric transfer of preference for an imprinting stimulus occurred in chicks. Moreover there was no difference between the hemispheres in uracil incorporation after 76 minutes' exposure in this type of bird. Intact birds were therefore no use in 'internal control' experiments.

Experiments by Meier (MEIER and CUÉNOD, 1970) on visual discrimination in pigeons in which the supraoptic commissure had been cut had shown that interhemispheric information transfer was minimal, so Horn et al (HORN et al, 1973) repeated the uracil incorporation experiments using chicks in which the supraoptic commissure had been cut. (There is a risk in this operation of cutting the anterior commissure also.) The birds were exposed to the stimulus for 60 minutes in four 15-minute periods with a 15-minute rest period in darkness between each exposure. Either the left or right eye was covered. The change from a continuous to a discontinuous exposure procedure was adopted because this latter method had been found to maintain the birds' responsiveness better over prolonged periods (BATESON - unpublished data). The birds taken for biochemical analysis were selected on the basis of showing no ill-effects due to surgery and a preference for the imprinted stimulus when tested with the exposed, but not the occluded, eye. Of 36 birds initially exposed only 12 met these criteria, although no

data is given as to the predominant reason for rejection. After comparing the uracil incorporation into presumed RNA between the trained and untrained sides of the three brain regions (forebrain roof and base, and midbrain) in each bird the only significant difference was a 15% elevation in the forebrain roof of the trained side of the brain i.e. contralateral to the trained eye, as compared to the untrained side. No differences were found between the trained and untrained sides in total radioactivity which Horn et al took as representative of the state of the precursor pool radioactivity. This is suspect on two grounds. Firstly their use of ^{3}H as isotope precluded any analysis of precursor pool radioactivity (see p. 7 above), indeed they criticised Metzger (METZGER et al, 1967) for his use of $^{3}H_2O$ in the same paper. Secondly they used a 150-minute precursor incorporation period so that, at most, the levels of total radioactivity which they found reflected no more than those at the time of killing, which immediately followed several bouts of handling and testing. Their categorical statement that: "(the absence of differences in total radioactivity) rules out the possibility that incorporation of the labelled base into macromolecules can be ascribed to assymmetric changes in pool size resulting, for example, from differences in cerebral blood flow . . . ", is manifestly unproven. Visual stimulation is known to influence cerebral blood flow (BONDY and MORELOS, 1971; BONDY et al, 1974), and this can occur to differential degrees in the two hemispheres. Thus the pool size during stimulation could have been different in the two hemispheres but not at the time of killing (analogously with the model suggested by TIPLADY,

1972 for whole brain in two different experimental situations). Only by making a series of observations throughout the exposure period can such effects be ruled out. In addition such differential blood flow could affect the extent of cellular interaction with hormones such as ACTH, because there would be more in the trained than untrained side of the brain. This is not to say that the result is therefore meaningless, because increased pool size may well be a necessary, but not sufficient, condition for increased synthesis of product (see HAMBLEY et al, 1974a). In this particular instance the use of ^{3}H-uracil makes the assessment of the real pool size of uracil very difficult, and as uracil is several metabolic steps removed from the final precursor of RNA, UTP, the pool specific activity of this latter is quite unknown.

An interesting by-product of these experiments was the observation that there was a right-left assymmetry in uracil incorporation rate in the operated, but not the intact, chicks. Horn et al point out that the operating procedure is itself assymmetric and may have been the cause of this effect, but no experiments have been performed to test this by reversal of the operating assymmetry.

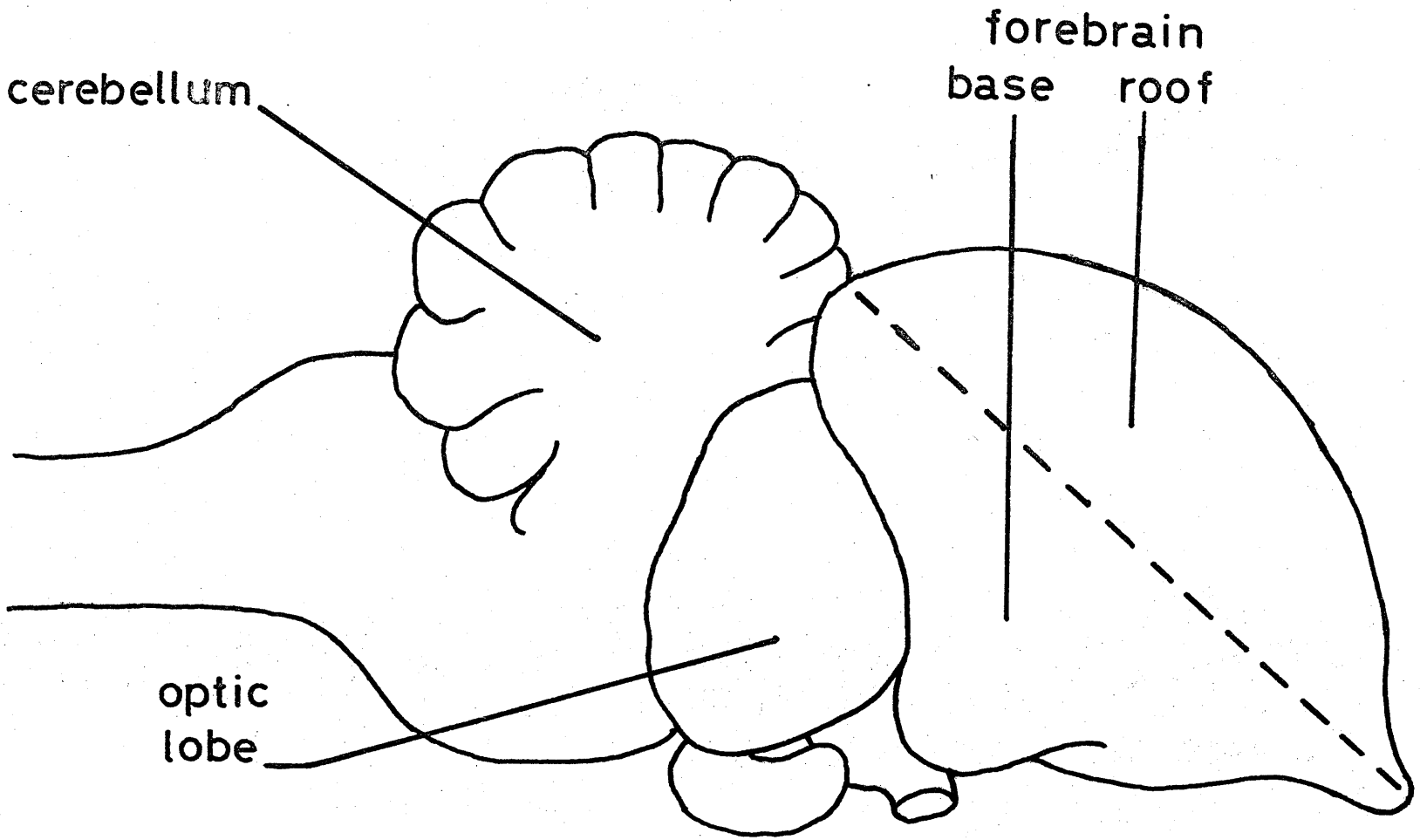
The changes in protein metabolism, preceded by similar ones in RNA metabolism, despite the difficulties of making detailed interpretations of them, were strongly suggestive of increased activity of the protein synthetic mechanism. However the measurement of the synthesis of these macromolecules by precursor injection remained only suggestive. If changes in some aspects of this metabolic process could be found which were independent

of such problems, and especially if these correlated well with the earlier results, this would support the hypothesis that new proteins (although not necessarily unique or previously unsynthesised types) were being formed as a consequence of the exposure of chicks to an imprinting stimulus. A likely candidate for such a study was DNA-dependent RNA polymerase (EC.2.7.7.6. nucleoside triphosphate - RNA nucleotidyl-transferase). Its activity had been reliably measured *in vitro* in cerebral nuclei from other species, and its characteristics were well defined. Thus, by assaying the activity of this enzyme under standardised conditions, the interpretative problems associated with the use of labelled precursors *in vivo* would be overcome. As expected from an enzyme which synthesises RNA its activity can be seen to increase before any increase in RNA is observed. This had already been demonstrated in eukaryotic cells in response to hormone stimulation (HAMILTON et al, 1968; TATA, 1968). Therefore not only could such changes in RNA polymerase activity, if found, support the interpretation of the uracil and lysine incorporation results, but the involvement of this enzyme would establish the existence of an earlier step in the process. It would then become possible to look for direct relationships between the action potential and the subsequent metabolic events leading to protein synthesis, if such relationships did indeed exist.

As necessary by-products of such an investigation there would be the development of a method for preparing nuclei from young chick brain, and the characterisation of DNA-dependent RNA polymerase in them, neither of which had been previously reported to have been done.

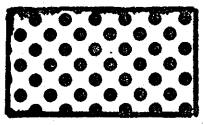
The following chapters describe the preparation of chick brain nuclei, the assay of RNA polymerase in them and the response of this enzyme after exposure of chicks to an imprinting stimulus.

FIGURE 3. Schematic representation of a sagittal section through the chick brain showing the dissection of the forebrain into roof and base portions (dotted lines).



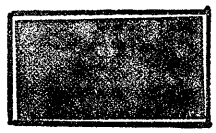
LEGENDS TO FIGURES 4-6.

Fig. 4 Schematic representation of the operations performed to expose chicks to either an imprinting stimulus, diffuse light or a dark-box. The birds were injected at the time shown with ^{3}H-lysine. At 160 minutes all birds were tested for 2 minutes before being killed. The temperature throughout was 30°C except for the test which was at 26°C.



stimulus

diffuse light



dark-box

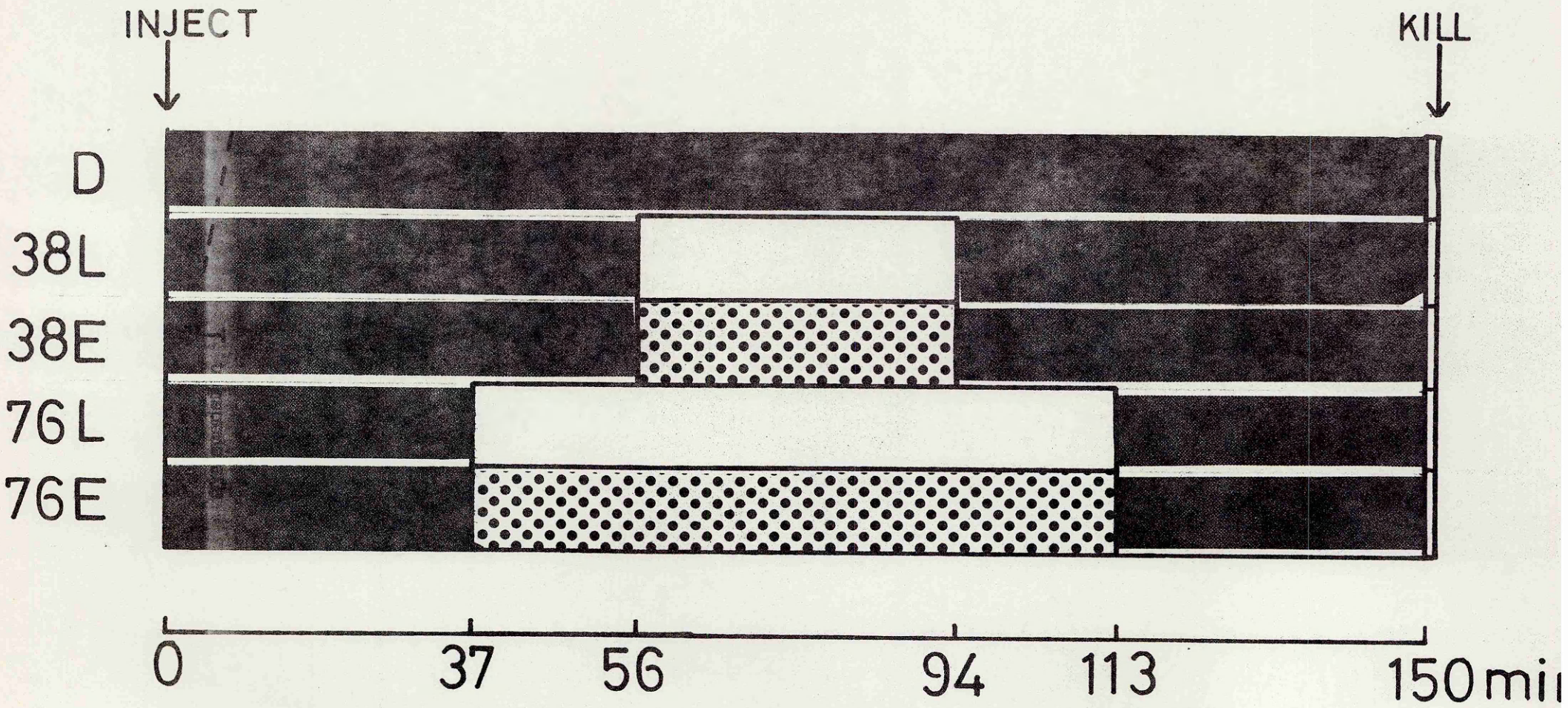
Fig. 5 Schematic representation of the operations performed to expose chicks to either an imprinting stimulus, diffuse light or a dark-box. The birds were injected with ^{3}H-uracil at the start of the experiment, tested for 2 minutes at the end and killed. The temperature throughout was 30°C, except for the test which was at 26°C.
The shadings are as for Fig. 4.

Fig. 6 Specific radioactivity (as % of the mean of the dark bird brain) of ^{3}H-uracil in the acid-insoluble material in three brain regions of chicks. The birds were exposed for 38 or 76 minutes to either an imprinting stimulus (solid lines), diffuse light (dotted lines) or a dark-box (time 0). Values are means ± s.e.m.

(Figures 4,5,and 6 are redrawn from BATESON et al, 1972)

FIG. 4

FIG. 5



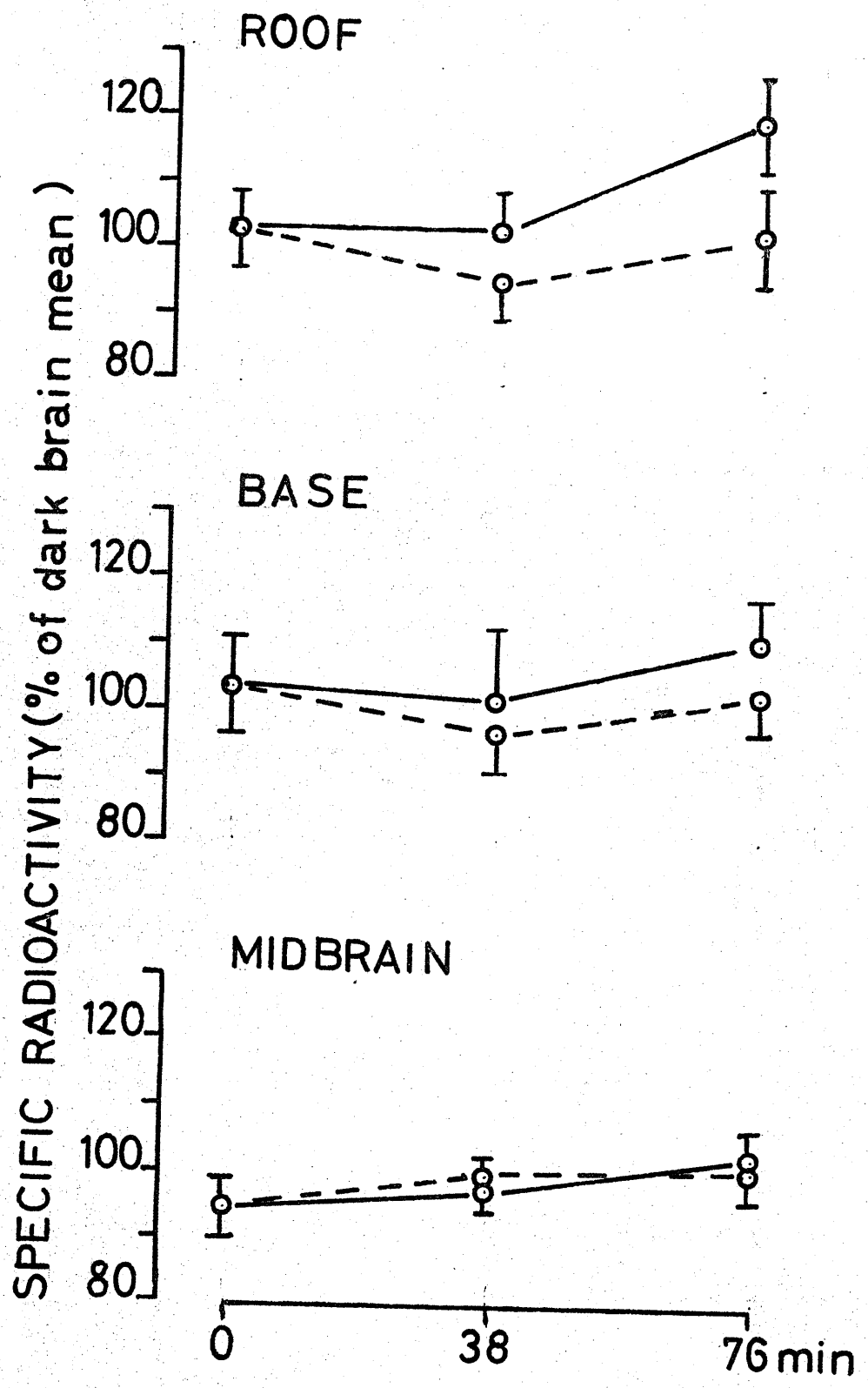

ROOF
120
100
80
BASE
120
100
80
MIDBRAIN
120
100
80
SPECIFIC RADIOACTIVITY (% of dark brain mean)
0
38
76 min

FIG. 6

CHAPTER III

It was anticipated that in future experiments which involved imprinting there would be twelve or more tissue samples which might need processing simultaneously. Thus a method of preparation of nuclei which could be easily handled by one person was required. Moreover because the number of chicks which could be treated from any one hatch was strictly limited (at best probably 24, most likely less) then pooling of samples was not possible. This meant that sufficient nuclei had to be obtained from a single brain region of the one-day old chick for triplicate RNA polymerase assay and DNA determinations, and so yield was an important criterion in assessing any preparation method. In addition the tissue, for at least the pilot experiment on the effects of exposure to an imprinting stimulus on cerebral RNA polymerase activity, would not be fresh but frozen in solid CO_2. This problem arose because the only equipment available for the exposure was at the Sub-Dept. of Animal Behaviour in Madingley, Cambridge, where there were no facilities for preparing nuclei and assaying their enzyme activity. Even in most of the later experiments the tissue would need to be frozen because the number of samples was likely to be greater than could be worked up in one day.

Most of the available methods for preparation of nuclei from brain had been worked out on fresh rat brain and were designed to maximise purity, even at the expense of yield. All previous experiments on the activity of RNA polymerase in cerebral nuclei were on the characteristics of the enzyme, not upon whether the enzyme activity altered in individual animals as a function of prior experience, and thus tissue could be pooled. For example,

the method used by Dutton and Mahler (DUTTON and MAHLER, 1968), modified from that of Widnell and Tata (WIDNELL and TATA, 1966) for rat cerebral cortex required a centrifugation of 3×10^6 g-min. and gave a yield of 350 µg DNA per g (wet weight) tissue, a percentage yield of approximately 30%. McEwen and Zigmond also used a 3×10^6 g-min. centrifugation and gained a DNA recovery of 45% (McEWEN and ZIGMOND, 1972). Both these methods, which are typical of the general preparation procedures for nuclei from brain, required at least one hour from start to finish even when only a few samples were used. Consequently for many samples the time needed would be much longer. Even accepting a long preparation time, the number of samples which can be prepared in most (if not all) ultracentrifuges is six; in many the number is three or four so that to prepare twelve samples of nuclei would require several 'runs'.

The method chosen for preparing the chick brain nuclei was a modification of that of Mertelsmann (MERTELSMANN, 1969) derived from an earlier source (HYMER and KUFF, 1964) which need centrifugations of only 2×10^4 g-min. and thus could be performed quite rapidly with up to 18 samples without excessive delays occurring at any stage.

Materials and Methods

Animals: Fertile eggs of a commercial broiler strain (Ross I, from Ross Poultry Products, Little Downham, Cambs.) were hatched in darkness at 40°C in an observation incubator (Curfew) and transferred, left in darkness at 33°C, in a brooder until their mean age was 24 hours.

Chemicals: Bovine serum albumin and yeast RNA type 11-S, β-mercaptoethanol and Tris-HCl were from Sigma Chemicals, London, U.K.

Calf thymus DNA was from BDH, Poole, Dorset as were all other reagents.

Water was de-ionised throughout.

Preparation of nuclei:

The birds were killed by decapitation at 24 hours post-hatch, their brains removed and dissected into forebrain roof, forebrain base and midbrain (Fig 3), the cerebellum being discarded. These samples were frozen in glass pots on acetone/solid CO_2 and stored at -20°C for two days so that they would be comparable with samples to be used in later behavioural experiments (Chapters V and VII). Each portion of tissue was allowed to warm up to 0-4°C in 4.5 ml of homogenisation medium with Triton X-100 (HMT). This consisted of 0.41 M sucrose; 0.4mM K_2HPO_4; 0.4 mM KH_2PO_4; 1 mM $MgCl_2$; 5mM β-mercaptoethanol and 0.5% w/v Triton X-100 at pH 6.5. The tissue was disrupted for 50 seconds at 1200 rpm by an M.S.E. top-drive macerator (Stage I). This suspension was then filtered under gentle vacuum through two layers of washed 100-gauge nylon bolting cloth (J. Stainier, Manchester) and the filtrate centrifuged at 2000 g-av for 10 minutes at 4°C in the 32 x 10ml angle head of an M.S.E. Mistral 4L. The supernatant was carefully decanted so that none of the pellet was lost, and discarded. The pellet was resuspended in 4.5ml homogenisation medium without Triton X-100 (HM) by five strokes of a hand-held, Teflon/glass Potter homogeniser (clearance: 200 μm) (Stage II). This suspension was centrifuged as before. The homogenisation medium HM thus removed virtually all the Triton X-100 from the preparation

and the total time the tissue was in contact with the detergent was at maximum 20 minutes. The supernatant from the second centrifugation was decanted, and the pellet resuspended as before in 4.5ml of Buffer A, which consisted of 10mM Tris-HCl pH 7.8, 10mM $(NH_4)_2SO_4$; 6mM $MgCl_2$; 1mM di-Na EDTA; 5mM β-mercapto-ethanol and 33% v/v glycerol (Stage III). This suspension was centrifuged at 1750 g-av for 10 minutes at 4°C and the pellet resuspended in 0.5mL of Buffer A by means of a vortex mixer. A flow diagram of this procedure is shown in Fig 7.

Determination of the purity of the nuclei: The nuclei were examined with a light microscope under phase contrast to check for debris and other contamination. The extent of this contamination by whole cells, and a count of the number of nuclei present was made using a haemocytometer. Electron micrographs of the nuclei were prepared using Spurr's Medium as embedding matrix and staining with osmium in Karnofsky fixative.

The DNA, RNA and protein content of the preparations were determined at each stage; DNA by the diphenylamine method (BURTON, 1956); RNA by the orcinol method (SCHNEIDER, 1945) and protein by the Folin-phenol method (LOWRY et al, 1951).

RESULTS

Under the light microscope the nuclei appeared to be virtually free of cytoplasmic contamination. Some capillary endothelial cells were always present in the preparations although at a level of 1:100 on an average cell to nucleus basis. This is somewhat higher than that found in nuclear preparations produced by

high speed centrifugation in concentrated sucrose solutions (Plates 1 - 4).

DNA, RNA and protein profiles

The nuclear preparation was monitored for its protein, RNA and DNA content at each stage during preliminary experiments. This was done on forebrain roof, forebrain base and midbrain separately rather than on the whole brain so that the average purity from each region would be known, in case the method was not equally applicable to all. No detailed histological data was available on the nuclear types found in each region, although the cell types are known to be quite heterogeneous (see for example The Avian Brain, Pearson), so no estimate could be made of any differential recoveries of nuclei of various sizes. However, it is likely that such effects would be less using this method than would occur with dense sucrose preparations which have critical dependence on such parameters as nuclear density (McEWEN et al, 1972). Also the relatively high yields in terms of DNA reduce such losses to some extent.

Tables 1,2,3 & 4 show the DNA, RNA and protein recoveries at each stage in the preparation (see Methods for details). There was appreciable variation in all these substances both between each batch of chicks and between chicks from the same batch, which could not be accounted for by variations in the assays. This is in accord with previous findings in chick brain (ZAMENHOF et al, 1971) where similar large variations were observed. These may be related to incubation conditions and to the fluctuations in the source of the eggs; the mother birds are not so strictly controlled genetically as are other standard laboratory animals where such large variations are not generally seen.

The DNA content of the tissue (on a µg DNA per mg wet weight basis) is comparable to that found by other authors, indicating that there were no gross discrepancies in the DNA assay procedure (ZAMENHOF et al, 1972; MARGOLIS, 1969), as was the total DNA content of the various regions.

Most of both the RNA and protein present in the initial homogenate (Stage I) was lost during the preparation, on average only 15% of the former and 4% of the latter remaining in the final nuclear suspension (Stage IV). The first centrifugation, in the presence of the detergent Triton X-100, removed the bulk of these (average 60% and 75% respectively). In a test experiment whole brain tissue was subjected to the procedure, but modified by the omission of triton X-100 from the medium; the protein contents of the preparations at Stage II and Stage IV were much higher (33% and 12% of Stage I respectively) and the nuclei at Stage IV were seen to have appreciable cytoplasmic adherents. This is entirely consistent with the known action of Triton X-100 which removes the outer nuclear membrane and consequently any cytoplasm which might be attached to it (LØVTRUP-REIN and McEWEN, 1966).

The ratios of DNA to both RNA and protein were different in the nuclear suspensions (Stage IV) derived from the forebrain roof and base as compared to that of the midbrain, namely there was less RNA but more protein in the former than the latter. This could reflect either or both of increased contamination and different preponderances of nuclear types. By visual inspection there was no consistent difference between them in terms of contamination which would argue against the former as a source of the variation. However the difference between them does lie within the experimental error so that the effect could be spurious.

The average DNA contents of individual nuclei from one-day old chick brain was estimated by relating nuclear number to the DNA content of the same suspension. This value was 2.81 $\pm$ 0.23 pg DNA per nucleus for all three brain regions, a figure in agreement with those of other authors (LESLIE, 1955).

DISCUSSION

The low-speed centrifugation method described was chosen as the standard method for producing nuclei fron one-day old chick brains for subsequent assay for RNA polymerase activity. It fulfilled the basic requirements of speed, ease of use by one person for the preparation of multiple samples and gave nuclei with reasonable purity in high yield. The cellular types from which these nuclei were derived, and their relative numbers is unknown. The criteria commonly used to distinguish the cellular source of nuclei from rat cerebral tissue may not be directly applicable to the neonate chick. Size is a risky criterion, even for the well-documented rat system, because it is closely related to the ionic composition of the medium (McEWEN et al, 1972). The presence of internal structures such as nucleoli have been extensively used as criteria for distinguishing nuclei of neuronal source from those of glial. The primary evidence for such identification has been taken from NURNBERGER, 1958. In rat most neuronal nuclei have a single, large nucleolus within a clear nucleoplasm which serves as a means of identification. The criteria for nuclei from other cell types are less well-defined. Moreover the possibility of changes in their internal structure during preparation makes such identification even more hazardous. For the neonate chick much less is known in terms of nuclear anatomy *in situ*, even less

in the case of isolated nuclei, thus rendering any estimates of the relative proportions of nuclear types in these preparations very difficult. On a crude estimation basis, taking only large nuclei (10 μm) with one or several discrete nucleoli (very few nuclei have only one nucleolus in chick brain) in clear nucleoplasm as being of neuronal origins, the percentage of nuclei in this category represented about 65% of the total in the Stage IV population.

Several reports have been published of fractionation procedures for preparing separate populations of cerebral nuclei 'enriched' in either neuronal or glial nuclei as compared to their relative frequencies in the initial homogenate (LOVTRUP-REIN and McEWEN, 1966; KATO and KUROKAWA, 1967; BURDMAN, 1970; AUSTOKER et al, 1972; THOMPSON, 1973). The neuronal populations were contaminated to varying extents with glial nuclei and the glial by neuronal. Moreover the large nuclei (i.e. 'neuronal') were probably a mixed population in themselves of neuronal and astrocytic nuclei, and the small nuclei (i.e. glial) probably included small neuronal plus endothelial nuclei. As expected from a fractionation procedure, the yield of nuclei (on a DNA basis) in each fraction was rather low and large starting quantities of tissue were needed. Lovtrup-Rein and McEwen achieved a total DNA recovery in the two fractions of 11%, Kato and Kurokawa 15%, and Thompson 20%. The same problems arise, but more acutely, in any attempt to fractionate the cerebral

nuclei from chick as were met in the purification of a heterogeneous population, namely yield and quantity of starting tissue. However, if only for the information which might be obtained on the relative activity of RNA polymerase and its characteristics in each nuclear type, if not for examining any changes in the enzyme after exposure to the imprinting stimulus, an attempt was made to fractionate these nuclei.

METHODS

Animals: These were incubated and reared as described earlier (p. 32).

Preparation of nuclei: Five fresh forebrain roofs were used. They were homogenised by five strokes of a Teflon/glass Potter homogeniser (clearance: 150 μm) at 800 rpm in 0.32M sucrose containing 1mM $MgCl_2$ to give a 30% (w/v) suspension. This was centrifuged for 10 minutes at 1000 gav and the pellet resuspended in approximately 5ml of either 2.0 or 2.2M sucrose containing 1mM $MgCl_2$. The DNA recovery at this stage was approximately 85%. A portion of this suspension was layered over a discontinuous sucrose gradient which had been left to stand at 4°C for 30 minutes to allow a small degree of interface diffusion to occur. This process tends to facilitate the passage of nuclei through the interfaces.

Two types of gradient were used (Fig 8); when the nuclei were resuspended in 2.0M sucrose they were layered over equal volume layers of 2.2, 2.4 and 2.8M sucrose (A), and when they were resuspended in 2.2M sucrose they were layered over equal volume steps of 2.4 and 2.8M (B) or 2.4, 2.6 and 2.8M sucrose (C). These gradients were made up in 5ml tubes and centrifuged in the 3 x 5ml swing-out head of an M.S.E. 75 centrifuge, at either 100,000

gav or 70,000 gav. The material at the interfaces was removed with a Pasteur pipette and suspended in 0.32M sucrose containing 1mM $MgCl_2$. These were examined by light microscopy under phase contrast and with cresyl violet staining.

RESULTS

The gradients used were similar to those used for fractionating mammalian cerebral nuclei but were not appropriate for the chick. Unlike the rat, no significant pellet was ever found to have sedimented through 2.8M sucrose, indeed, the vast bulk of the nuclei did not pass through the first interface (2.0/2.2 in the case of gradient A; 2.2/2.4 in the case of gradients B and C). The relative proportions of presumed neuronal and glial nuclei were determined by counting in a haemocytometer; large nuclei with clear nucleoplasm and one or two distinct nucleoli were counted as neuronal and small nuclei with relatively dense nucleoplasm were counted as glial. On gradient A, after centrifugation at 70,000 gav for 60 minutes, the proportions of neuronal to glial nuclei at interfaces 2 and 3 were as shown in Table 5 . On gradient C, after centrifugation at 70,000 gav for 60 minutes, the relative proportions at interfaces 2 and 3 were as shown in Table 5 . No counts were made on nuclei from gradient B because no significant pellet was obtained at the bottom of the tube and interface 1 was very heavily contaminated with debris, thus only one interface, number 2, had clean nuclei in it.

Similarly with the results of other reports the glial nuclei were much less contaminated than the neuronal, presumably because whereas small nuclei may remain in low density sucrose, large ones are unlikely to penetrate high density sucrose. This

is supported by a comparison of the nuclei at interface 3 in both gradients. Gradient B, with 2.4M sucrose above the interface has far fewer large nuclei at this interface than gradient A with 2.2M sucrose above the interface. Also at interface 2, in the denser gradient B the proportion of large nuclei found here was smaller than found in gradient A. Even increasing the time of centrifugation from 60 to 90 minutes had little effect on this distribution. Thus the best 'neuronal-enriched' population was still 35% contaminated with glial nuclei, and the total DNA recovery from the two interfaces combined was only 14%.

These results indicated that the difficulties inherent in trying to produce a method for preparing neuronal and glial nuclei from the chick brain, which could be used on tissue from behavioural experiments, were likely to be very great. Consequently the attempt was abandoned.

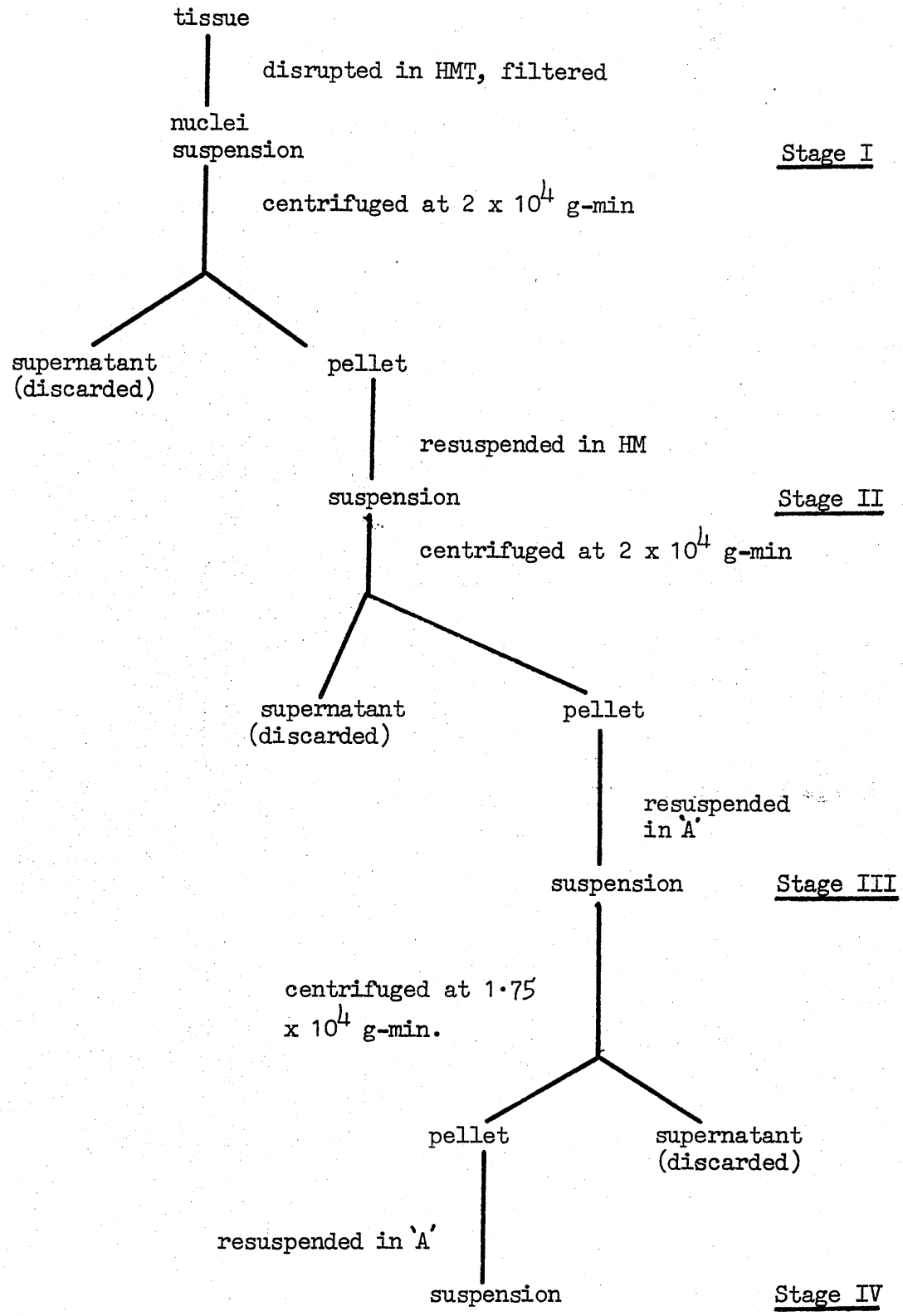
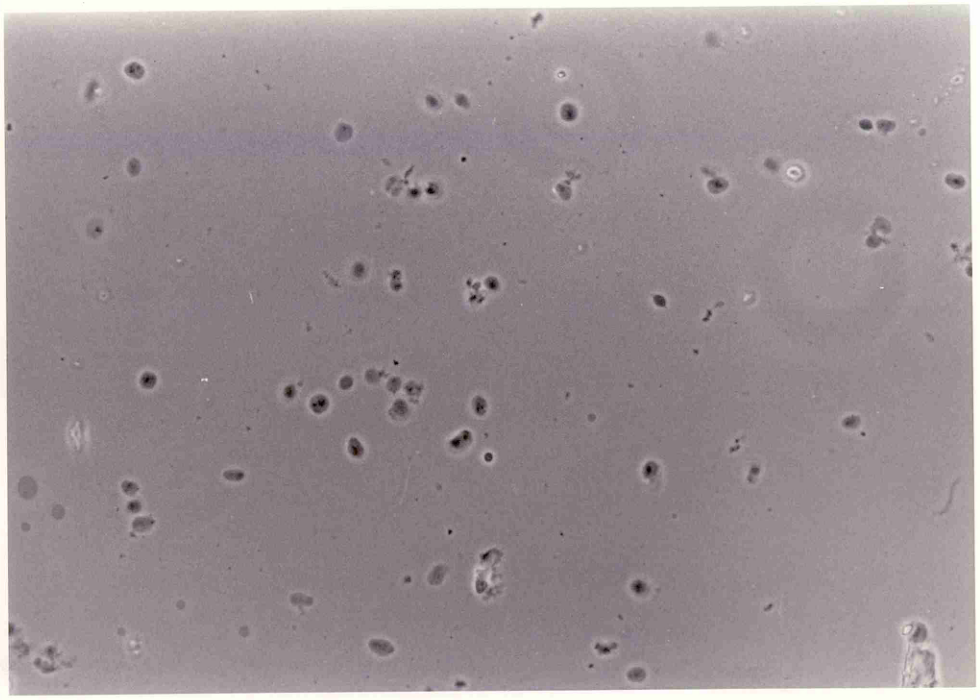
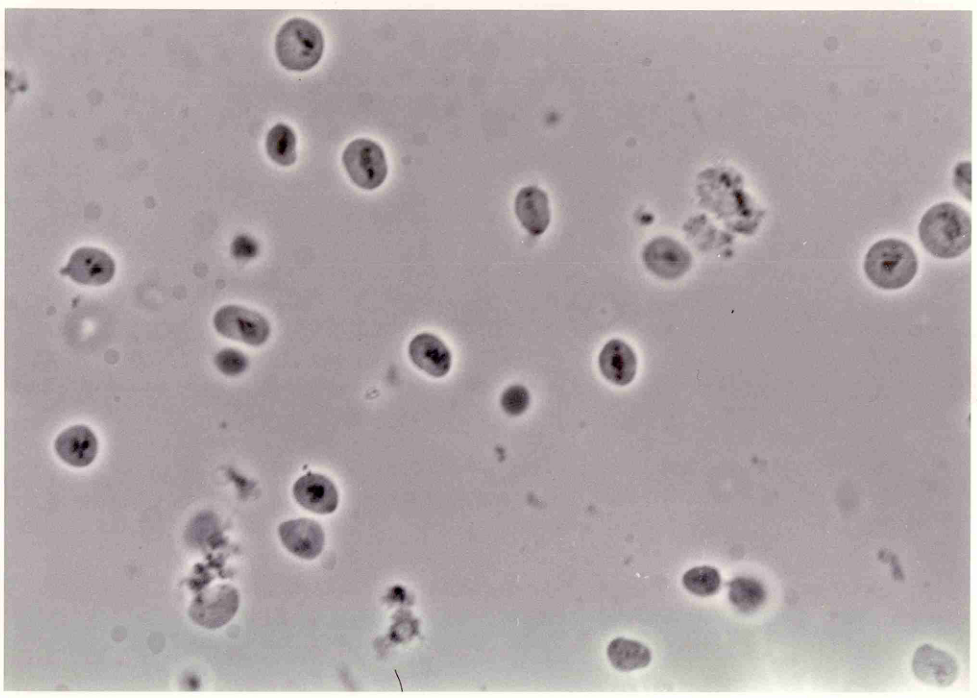


FIGURE 7. Flow diagram of the steps involved in the preparation of chick brain nuclei. See Methods for details.

1.

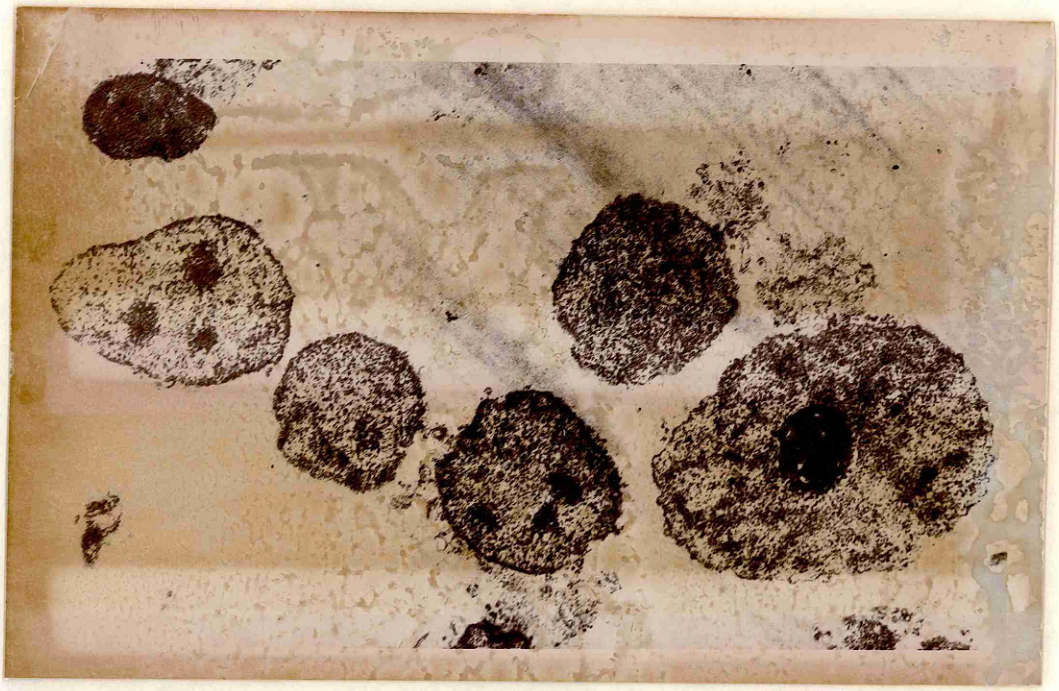


2.



PLATES 1 and 2.

Chick brain nuclei from Stage IV suspension viewed under phase contrast. See Methods for details. Magnification: 1: 240×
2: 640×



3.

4.

PLATES 3 and 4.

Chick brain nuclei from Stage IV suspension stained with uranyl acetate. See Methods for details. Magnification: 3: 7.5×10^3 X

4: 30×10^3 X

TABLE 1

Typical DNA content
and recoveries in preparations of nuclei
from three regions of chick brain

DNA content (μg/mg fresh wt.)

	Brain region		
Stage	Roof	Base	Midbrain
I	1.08 ± 0.09	1.04 ± 0.09	1.22 ± 0.13
II	1.04 ± 0.14	1.01 ± 0.16	1.18 ± 0.19
III	0.99 ± 0.15	0.90 ± 0.16	1.12 ± 0.21
IV	0.87 ± 0.18	0.78 ± 0.20	1.01 ± 0.22

DNA recovery (as % of Stage I)

I	100	100	100
II	96.3 ± 5.4	97.1 ± 7.6	96.7 ± 5.9
III	91.7 ± 7.0	86.5 ± 8.6	91.8 ± 8.3
IV	80.6 ± 10.9	75.0 ± 13.9	82.8 ± 10.3

DNA content was assayed at each stage of the preparation (see Methods for details). Values are mean ± range ($N \leq 8$).

TABLE 2

Typical RNA content
and recoveries in preparations of nuclei
from three regions of chick brain

RNA content (μg/mg fresh wt.)

Stage	Brain region: Roof	Base	Midbrain
I	2.77 ± 0.36	3.04 ± 0.38	2.78 ± 0.31
II	0.93 ± 0.15	1.29 ± 0.19	1.18 ± 0.19
III	0.46 ± 0.09	0.51 ± 0.13	0.63 ± 0.14
IV	0.38 ± 0.10	0.39 ± 0.14	0.54 ± 0.17

RNA recovery (as % of Stage I)

I	100	100	100
II	33.6 ± 1.3	42.4 ± 1.0	42.5 ± 2.4
III	16.6 ± 1.2	16.8 ± 2.5	22.7 ± 2.9
IV	13.7 ± 2.1	12.8 ± 3.5	19.4 ± 4.4

RNA content was assayed at each stage of the preparation (see Methods for details). Values are mean ± range. ($N \leq 8$)

TABLE 3

Typical protein content
and recoveries in preparations of nuclei
from three regions of chick brain

Protein content (μg/mg fresh wt.)

	Brain region		
Stage	Roof	Base	Midbrain
I	82.6 ± 6.6	84.1 ± 5.9	80.3 ± 6.0
II	16.9 ± 3.3	19.8 ± 3.5	21.9 ± 4.2
III	10.1 ± 2.4	13.0 ± 2.3	13.2 ± 2.2
IV	3.0 ± 1.3	3.4 ± 1.5	3.6 ± 1.6

Protein recovery (as % of Stage I)

I	100	100	100
II	20.5 ± 2.1	23.6 ± 2.3	27.4 ± 2.9
III	12.2 ± 1.8	15.4 ± 1.6	16.4 ± 1.4
IV	3.6 ± 1.2	4.0 ± 1.4	4.5 ± 1.5

Protein was assayed at each stage of the preparation (see Methods for details). Values are mean ± range. ($N \leq 8$).

TABLE 4

Typical relative DNA, RNA and protein contents of nuclear preparations from chick brain at Stages I and IV

DNA : RNA (µg DNA/µg RNA)

	Brain Region		
Stage	Roof	Base	Midbrain
I	0.39	0.34	0.44
IV	2.29	2.00	1.87

DNA : protein (µg DNA/µg protein)

	Brain Region		
Stage	Roof	Base	Midbrain
I	0.01	0.01	0.02
IV	0.29	0.23	0.28

DNA, RNA and protein were assayed as described in Methds. Data derived from Tables 1, 2 and 3. ($N \leq 8$)

TABLE 5.

Relative proportions of "neuronal" and "glial" nuclei obtained from interfaces 2 and 3 of two sucrose gradients

Interface	Gradient Type A	Gradient Type B	Nuclear Type
2	65 ± 5	58 ± 6	Neuronal
	35 ± 5	42 ± 6	Glial
3	14 ± 7	10 ± 2	Neuronal
	86 ± 7	90 ± 2	Glial

(Both gradients were prepared as described in Methods. Values ± are percentages of total nuclei at each interface - range. N ≤ 7)

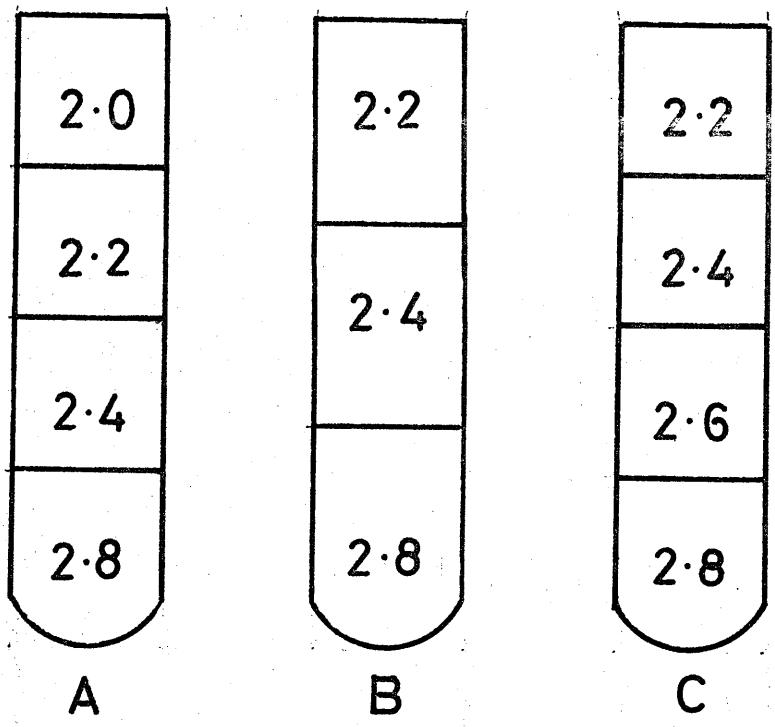


FIGURE 8. The three types of discontinuous sucrose gradients used in the attempt to fractionate chick cerebral nuclei into neuronal and glial types. Numbers indicate the concentration (M) of the sucrose in each step.

CHAPTER IV

DNA-dependent RNA polymerase has been assayed *in vitro* in nuclei from many tissue sources. Although the fine details of its response to its assay conditions vary somewhat, its general characteristics, in nuclei from mammalian sources, are very similar, in particular its response to the presence and concentration of divalent metal ions and to the ionic strength of the medium. There are distinct optima for the concentrations of both magnesium (Mg^{2+}) and manganese (Mn^{2+}), and usually higher concentrations of the former are required to attain maximal activity. Increasing the ionic strength of the assay medium, either by the addition of potassium chloride or ammonium sulphate causes several-fold increases in the activity with maximal activation at around 100 mM and 300-400 mM respectively. In rat liver nuclei it was proposed that there were two enzyme forms on the basis of the response of the enzyme to these different conditions, one with preference for Mg^{2+} at low ionic strength (form I) and the other for Mn^{2+} at high ionic strength (form II). Autoradiographic data and the separation of the constituents of the nucleus (nucleolus and nucleoplasm) showed that these two forms did indeed exist; form I appeared to be localised in the nucleolus and form II in the nucleoplasm. By measuring the relative proportions of the four nucleotide bases in the products formed by the *in vitro* reaction (base ratios) form I was shown to produce a ribosomal-like RNA and form II a DNA-like RNA (probably messenger RNA). (WIDNELL and TATA, 1966; MAUL and HAMILTON, 1967; ROEDER and RUTTER, 1969, 1970.)

RNA polymerase in nuclei derived from brain tissue was found to have similar divalent ion and ionic strength requirements (BARONDES, 1964; DUTTON and MAHLER, 1968; DRAVID and DUFFY, 1969; BANKS and JOHNSON, 1973). However in all these studies a heterogeneous population of nuclei were used, that is no attempt was made to define their type of cell source. When methods for fractionating nuclei according to size were devised it was possible to ascribe the characteristics of the enzyme in them to those of the cells from which they were presumed to derive, i.e. neuronal or glial (see however Chapter III on the difficulties of such an assignment). The presumed-neuronal nuclei had higher RNA polymerase activity than the presumed-glial nuclei but did not differ greatly with respect to optimal assay conditions (LØVTRUP-REIN and McEWEN, 1966; KATO and KUROKAWA, 1967 and 1970; SLAGEL and AKERS, 1972).

Although no reports of studies on the two enzyme forms in fractionated nuclei has appeared, recent results on the use of α-amanitin, which extensively inhibits the activity of form II, may prove useful for avoiding the need to separate nucleoli from nucleoplasm (BANKS and JOHNSON, 1973).

In the light of the earlier research into the characteristics of DNA-dependent RNA polymerase in cerebral nuclei and the absence of any information for the chick brain, it was necessary to determine the optimal conditions for the assay using nuclei prepared as described in Chapter III. In addition, the majority of previous work has used adult tissue and so it was possible that the enzyme in nuclei from a rapidly developing neonate animal, like the one-day old chick, could be markedly different.

Such a study was a precondition of any future studies using chicks exposed to the imprinting stimulus.

Materials and Methods:

Chemicals Sigma Chemical Co. London, U.K. : ATP, CTP, GTP and UTP; β-mercaptoethanol; calf thymus DNA (type 1); calf thymus DNase; Actinomycin D; Tris-HCl.

Radiochemical Centre, Amersham, U.K. + (8-14C)-ATP specific activity 40 mCi/mmol; 14C(U)-CTP 405 mCi/mmol; 14C(U)-GTP 450 mCi/mmol; 14C(U)-UTP 405 mCi/mmol.

BDH Poole, U.K. : Butyl-PBD (2-(4'tert.-butylphenyl)-5-(4" biphenylyl)-1,3,4-oxadiazole) and all other reagents, which were of analar grade.

One-day old chick brain nuclei were prepared by the method described in Chapter III and kept at 0-4°C until assay, which was within 1.5 hours of their preparation. They were suspended in Buffer A except in the case of experiments which involved variations of the concentrations of ammonium sulphate, magnesium or manganese when these were omitted from the suspending buffer.

The RNA polymerase incubation medium was very similar to that used by Kato and Kurokawa and consisted of: 100mM-Tris HCl pH 8.5; 300mM-$(NH_4)_2SO_4$; 5mM-$MgCl_2$; 5mM-β mercaptoethanol; 0.5mM concentration of each of CTP, GTP and UTP; 0.1mM ATP; 0.167 μCi of (8-14C)-ATP. This medium gave maximal activity and was the one used unless otherwise stated. 400 μl of incubation medium were heated at 37°C for 1 minute; 100 μl of nuclear suspension (containing approximately 8 x 10^6 nuclei - 30 μg DNA) was added, mixed and the mixture incubated

for a further 5 minutes at 37°C. At the end of this period the reaction was stopped by the addition of 0.5 ml ice-cold 10% trichloroacetic acid (TCA) containing 0.2M sodium pyrophosphate. The tubes were kept in ice for 20 minutes to allow the precipitate to coagulate and the contents of the tubes were then filtered through 2.5cm Whatman GF/C glass fibre filters by applying gentle vacuum. The filters were successively washed at room temperature in the equivalent of 25ml 5% TCA, 25ml chloroform/ethanol (3:1) and 15ml ether and dried. The filters from one experiment were washed simultaneously in the appropriate reagent by placing each filter from a triplicate assay in a numbered Teflon holder (Fig 9). The enzyme controls, produced by incubating the nuclei in the presence of 10% TCA plus 0.2M sodium pyrophosphate, were found to be almost identical to each other and so the several control tube filters were grouped together in holders without identification. The holders were placed for a few minutes in 5l tanks containing a volume of each reagent (replaced for each experiment) equivalent to either 25ml or 15ml per filter, and were stacked so as to allow free access of the reagents to the filters. They were removed from the TCA tank to the chloroform/ethanol tank, to the ether tank and were then left to dry at room temperature. This procedure not only greatly speeds up the washing process and makes it more uniform but prevents the loss or misplacing of the filters (up to 60 per experiment) during the manipulations. There was no difference in radioactivity between the control filters produced by this method and those produced by individual washing.

The dry filters were placed in scintillation vials, 10ml of scintillation fluid (8g/l Butyl P-BD in 1:1 toluene-methoxyethanol) added, and the radioactivity counted in a Beckman LS-150 liquid scintillation spectrometer. The efficiency of counting of ^{14}C was 85%. To ensure that there was no appreciable internal absorption of radiation in the filters, tests were run in which half the filters were counted in the above way and the other half were digested for 10 minutes at 100°C in 1M NaOH. The digested samples were counted in the above scintillant, corrected to the same efficiency and compared to the filter samples. As expected the digested samples had apparently higher radioactivity, the ratio filter/digest being 0.91 ± 0.05 (N = 12). All subsequent radioactivities were corrected to compensate for this effect.

To determine that the incorporation of ^{14}C-ATP was the result of the action of DNA-dependent RNA polymerase the reaction was carried out in the presence of either actimomycin D which is known to inhibit the action of the enzyme, or DNase to degrade the endogenous DNA template. The actimomycin D was added at a concentration of 50 µg/ml to the incubation mixture at zero time and the assay carried out as usual. The DNase (50 µg/ml) was added to the nuclei and incubation medium from which the nucleotide triphosphates (NTP) was omitted. They were incubated for 5 minutes at 37°C before the NTPs were added and the reaction continued as above. Parallel reactions were run without DNase.

To examine the response of the enzyme to different divalent metal ion concentrations, pH and ionic strength the assay

medium was prepared by combining its several constituents as required. Thus to produce assay media with different magnesium concentrations the standard medium was made up without magnesium and this latter was added in the form of a concentrated solution to the desired final concentrations.

Results

There were substantial differences in the activity of RNA polymerase between nuclei prepared from chicks drawn from different hatches, and generally much smaller differences between those drawn from the same hatch (Tables 6 and 7).

TABLE 6

Variation in RNA polymerase in the brains of chicks drawn from different hatches

	Day 1	Day 4	Day 6
Hatch 1	3860	3510	
Hatch 2		3300	3140
Hatch 3			4770

Mean RNA polymerase activity (pmol ATP incorporated/mgDNA/5 minutes)in three hatches of chicks. Each value is the mean of 6 determinations. Each brain was divided in half antero-posteriorly, and nuclei prepared from each half on the days shown above. All the tissue had been frozen at least two days before its first assay, i.e. for Hatch 1, Day 1 was after 2 days of frozen storage.

TABLE 7

Variation of RNA polymerase specific activity in the brains of several chicks from two hatches

	Hatch 1	Hatch 2
	4190	5120
	3930	4970
	3910	4910
	3820	4840
	3700	4400
	3620	4390
mean ± sem	3860 ± 80	4770 ± 120

The specific activity of RNA polymerase in nuclei prepared from the brain of several chicks drawn from two separate hatches (the hatches are the same as those used for data in Table 6). Values are pmol ATP incorporated/mg DNA/5 minutes. Assay as in Methods.

Similar observations have been reported in rabbit and monkey brain (BONDY and WAELSCH, 1965). That this difference was not due to variations in the preparation of the nuclei nor in the assay was ascertained by simultaneously assaying nuclei from the brains of chicks drawn from separate hatches. The slight loss of activity caused by storage of the frozen brains from one hatch until material from the next hatch was ready (approximately 10%) did not seriously affect this comparison. To counteract this effect, which would otherwise have introduced a large scatter into the data, the average RNA polymerase specific activity was calculated from the data of several nuclear preparations from birds of several hatches. In all subsequent experiments at

least one standard assay (this is, as described in Methods above) was made and all the data from the experiments were normalised to the previously determined average value by means of the standard assay. This average value was 4000 pmol ATP incorporated/ mgDNA/5 minute incubation.

In experiments where several non-imprinted birds were used, for example the experiments described in this Chapter, all the nuclei were pooled and thus any variations in activity between birds were eliminated.

The choice of a 5-minute incubation time was made on the basis of the data shown in Fig 10. The reaction was linear for just over 5 minutes, and then began to level off quite quickly. Activity profiles like this have been seen by several other authors although the period of linearity varies with the preparation and assay procedures and the source of the nuclei (BARONDES, 1964; BONDY and WAELSCH, 1965; KATO and KUROKAWA, 1970; BANKS and JOHNSON, 1973). Both loss of enzyme activity and the action of RNases on the product probably contribute to the decline in activity. After 5 minutes of incubation the incorporation of ATP per mg DNA present was calculated from the known specific activity of the exogenous ATP. If however the level of endogenous ATP was a significant proportion of the added ATP then the specific activity of the ATP calculated would be too high and the estimated activity of RNA polymerase would be an under-estimate. Thus the figures given are the minimum RNA polymerase activities. The maximal value of approximately 800 pmols ATP incorporated/mg DNA/minute is substantially higher than that found by others, e.g. Bondy and Waelsch in rat brain

nuclei - 200 pmols ATP/mg DNA/minute; Kato and Kurokawa in guinea pig neuronal nuclei (P_{51} type) 200 pmols ATP/mg DNA/minute. Both these determinations were on nuclei from adult tissue, and higher activities might have been expected in the young from observations made in the developing mouse brain (BANKS and JOHNSON, 1972).

Response to Inhibitors

That the incorporation of ^{14}C-ATP was dependent on the presence of DNA and that the formation of product could be mostly prevented by the presence of actinomycin D is shown in Table 8. These results are exactly as expected if the enzyme activity measured was DNA-dependent RNA polymerase.

TABLE 8

Effects on RNA polymerase activity of Actinomycin D and DNase

	pmol ATP incorporated/ mg DNA/5 minutes	% inhibition over control
Actinomycin D	+ 222 ± 44	94
	- 3808 ± 242	0
DNase	+ 389 ± 69	87.5
	- 3119 ± 146	0

Effects of actinomycin D (50 µg/ml) and DNase (50 µg/ml) on the incorporation of ^{14}C-ATP into RNA by chick brain nuclei. Values are mean ± sem. N = 12. (Incubation details as in Methods.)

The 20% loss of activity in the control in the DNase experiments compared to the control in the Actinomycin D experiments (both were performed using the same nuclear preparation) was possibly

due to the 5-minute pre-incubation of the nuclei with DNase. This suggests that the enzyme is rather unstable at 37°C in the incubation medium, although at 0-4°C in Buffer A the activity loss after 1 hour was only about 8%.

Response to DNA concentration

The incorporation of ATP was linear with respect to enzyme concentration, measured as DNA content, over the whole range ever likely to be used in these experiments (Fig 11). To obtain DNA concentrations of 60 µg/100µl required the pooling of nuclei from several brains; the concentration of DNA from one brain region was approximately 25-30 µg/100 µl. This was considerably less than the DNA content per assay used by other authors, e.g. Banks and Johnson - up to 200 µg DNA; Kato and Kurokawa - 140 µg DNA and Dutton and Mahler - 150 µg DNA, who achieved their higher amounts by pooling tissue from several animals. It was not possible in the later behavioural experiments to use pooling of tissue to increase the absolute amount of ATP incorporation achieved although in the experiments described in this chapter this was done.

Response to pH

The pH of the incubation medium was varied by adjusting the pH of the Tris buffer and of the ammonium sulphate. The activity of the enzyme was measured at each pH value (Fig 12) and in accordance with the findings of others (WIDNELL and TATA, 1966; DUTTON and MAHLER, 1968), there was a broad range of pH which gave maximal activity. The pH used in the standard assay medium (8.5) was within this range.

Response to divalent metal ions

In common with the findings of all other authors there was a difference in the enzyme's response to the concentration of different divalent metal ions. Fig 13 shows the effect on the incorporation of ATP of increasing concentrations of magnesium, at both high and low ionic strength, and Fig 14 shows the effects of manganese under the same conditions. The presence of 300 mM ammonium sulphate (high ionic strength) increased the activity of the enzyme, at optimal divalent metal ion concentration, by three- to four-fold. The minimum optimal concentrations were 5mM for magnesium and 2mM for manganese. For manganese this was a true optimum, the activity being lower below and above 2mM. All concentrations from 2mM to 10 mM magnesium were optimal at high ionic strength, at low ionic strength the optimal concentration was 2mM and above. The absence of any divalent metal ion reduced the activity of the enzyme virtually to zero. The activity was approximately equal in the presence of 5mM Mg^{2+} or 2mM Mn^{2+}.

In 3-day old mouse brain nuclei BANKS and JOHNSON (1973) found approximately equal activity with optimal concentrations of either Mg^{2+} or Mn^{2+}, the optima being 5mM and 0.5mM respectively. These assays were in the absence of ammonium sulphate but did contain 70 mM KCl. The addition of 100 - 150 mM ammonium sulphate increased the activity of the enzyme twofold. The profiles of magnesium and manganese activation curves were similar to those shown in Figs 13 and 14, namely a very low activity in the absence of divalent ion, rising, very sharply in the manganese case, to a peak at optimal concentration and then

decreasing, and more gradually, reaching a plateau, in the magnesium case.

Neuronal nuclei from adult guinea pig cortex also were found to exhibit similar characteristics to those described here (KATO and KUROKAWA, 1970). The magnesium and manganese activation curves at high ionic strength (300mM ammonium sulphate) had profiles as shown in Figs 13 and 14 with optima at 5mM and 2mM respectively. However the maximal activity in the presence of 2mM Mn^{2+} was 3 times higher than that in the presence of 5mM Mg. This differs quite markedly from the findings of Banks and Johnson, even in adult mouse, where the Mn^{2+}-stimulated activity was slightly greater than the Mg^{2+}-stimulated activity.

Response to ionic strength

The variation in activity of the chick brain enzyme with varying ionic strength when stimulated by Mg^{2+} (5mM) was very little different to that in the presence of Mn^{2+} (2mM) (Fig 15). Optimum ionic strength was provided by 300 mM NH_4^+ for the Mg^{2+} reaction and 400 mM NH_4^+ for the Mn^{2+} reaction, and there was only an approximately 10% difference in activity between the two at their respective optimal ionic strengths ($\frac{Mg^{2+}}{Mn^{2+}} = 1.10$).

Other authors have used either or both of potassium chloride or ammonium sulphate to create high ionic strength in the assay medium. In general quite close agreement has been found between nuclei from different mammals in the response of RNA polymerase to such conditions of assay.

Using NH^{4+} alone to generate high ionic strength, KATO and KUROKAWA (1970) found that for neuronal nuclei from guinea pig

300 mM NH_4^+ was optimal for Mn^{2+} and 450mM for Mg^{2+}, with an Mn^{2+}/Mg^{2+} ratio of 2 at their respective maxima. Also using only NH_4^+, DRAVID and DUFFY (1969) found rat cortex nuclei most active in the presence of 300 mM NH_4^+ (with 4mM Mn^{2+}) and in rat liver WIDNELL and TATA (1966) found 410mM optimal in the presence of either Mg^{2+} or Mn^{2+}, the ratio Mn^{2+}/Mn^{2+} being 3.

The 'mixed-ion' systems, essentially based upon the assay of DUTTON and MAHLER (1968) for low ionic strength, have used 70mM KCl plus increasing concentrations of NH_4^+. For instance the assay of BANKS and JOHNSON (1973), using mouse brain nuclei, required 50-200mM NH_4^+ for maximal activity in the presence of Mg^{2+} and 150mM NH_4^+ in the presence of Mn^{2+}. The ratio of Mn^{2+}/Mg^{2+} was 1.0-1.3. In sheep glial nuclei, SLAGEL and AKERS (1972) using 70mM KCl and 8mM Mg^{2+}, found that 200 mM NH_4^+ gave maximal enzyme activity. Thus although there was a complex relationship between ionic strength generated by KCl and NH_4^+, it would appear that higher NH_4^+ concentrations are needed in the absence of KCl than in its presence.

Relative rates of RNA and poly A synthesis

The presence of homopolyribonucleotide polymerases in mammalian nuclei is well-known (MANDEL, DRAVID and PETE, 1967; KATO and KUROKAWA, 1970), poly-A polymerase and poly-C polymerase having the highest activities. Although the problem of their contribution to the total labelled nucleotide incorporation can be largely avoided by the use of labelled GTP or preferably UTP the expense of the assay is appreciably increased. For an extensive program of assays this is of some importance,

so the contribution of poly-A polymerase to the apparent activity of chick brain RNA polymerase in this system was measured under various conditions of assay.

The incorporation of ATP into product in the absence of CTP, GTP and UTP is shown in Table 9, and for comparison the incorporation in the presence of the other NTPs.

TABLE 9

Relative activities of poly-A and RNA polymerases in chick brain nuclei

	+ 5mM Mg^{2+}	+ 2mM Mn^{2+}	
+ NH_4^+	83	210	+ ATP
	3825	3131	+ NTP
- NH_4^+	59	123	+ ATP
	1628	1380	+ NTP

All results are in pmol ATP incorporated/mg DNA/5 minutes. All assays as described in Methods except that Mg^{2+}, Mn^{2+} or NH_4^+ were deleted as necessary. ATP alone was at a concentration of 0.5mM; NTP was ATP, CTP, GTP and UTP each at a concentration of 0.5mM.

In the presence of 300 mM NH_4^+ the poly-A polymerase reaction was only 2% as great as that of the Mg^{2+}-activated RNA polymerase reaction and only 7% as great as the Mn^{2+}-activated RNA polymerase. Even these values are likely to be an overestimate of the extent of poly-A function in the RNA polymerase assay. As Kato and Kurokawa have shown, the presence of the three other NTPs inhibits the formation of poly-A . Thus the true activity of the RNA polymerase is not appreciably confounded by the action of poly-A polymerase in the assay as described here.

Relative incorporation of NTPs

To make a simple estimation of the type of RNA being produced by the action of the nuclear RNA polymerase the incorporation of each of the nucleotide triphosphates was measured in the presence of the other three. This was done by adding 0.25 μCi of ^{14}C-labelled ATP, CTP, GTP or UTP to the reaction medium, the concentrations of all the NTPs being equal. Thus the specific activities were equal as the contribution to the concentration of its NTP by the labelled NTP was less than 2%. The results of the experiment are shown in Table 10, with the incorporation ratios of other authors for comparison.

TABLE 10

Relative incorporations (%) of nucleosides into the RNA formed by RNA polymerase *in vitro* in several tissues

Tissue (ion)		Nucleoside: Adenosine (A)	Cytidine (C)	Guanosine (G)	Uridine (U)	Base Ratio $\frac{(A + U)}{(G + C)}$
guinea pig cortex[1]	(Mg)	22.8	24.3	31.9	21.0	0.779
	(Mn)	22.0	23.8	32.7	21.4	0.768
rat cortex[2]	(Mn)	29.9	25.4	30.0	14.7	0.805
rat liver[3]	(Mn)	24	26	23	27	1.040
rat liver[4]	(Mn)	15.8	31.3	26.7	26.3	0.726
	(Mg)	13.4	29.8	30.9	26.0	0.649
rat liver[5]	(Mn)	25.4	22.4	23.5	28.8	1.190
chick brain[6]	(Mg)	15.8	25.4	28.4	30.1	0.848
	(Mn)	13.9	24.9	30.9	30.7	0.805

Source: 1 KATO and KUROKAWA, 1970
2 DUTTON and MAHLER, 1968
3 WIDNELL and TATA, 1966
4 BEATO et al, 1970
5 POGO et al, 1967
6 this study

The $\frac{A + U}{G + C}$ ratio of DNA is approximately 1.4 and of ribosomal RNA, 0.6 in rat brain (JACOB et al, 1966), thus the RNA synthesised by the chick brain nuclei appears from its $\frac{A + U}{G + C}$ ratios to be predominantly ribosomal, although the higher ratio than many other studies suggests the presence of some mRNA. However the percentage incorporation of each isotope varied quite markedly from report to report. For instance the ratio $\frac{A + U}{G + C}$ from Dutton and Mahler's work (1968) was 0.805 and the ratio A/U was 2.0. In this present work the ratio $\frac{A + U}{G + C}$ was also 0.805 in the presence of Mn^{2+} but with an A/U ratio of 0.45, a complete reversal of the relative amounts of ATP and UTP incorporated. This effect may be due to differences in the base composition of the DNA being transcribed or to other unidentified features of the assay procedure, e.g. high ionic strength effects which can alter the base ratio of the RNA synthesised (POGO et al, 1967). In the absence of further information as to the mechanism of this effect it is not possible to make firm suggestions as to the type of RNA synthesised by the chick brain nuclei under these conditions.

General Discussion

The activity of RNA polymerase *in vitro*, as measured by the assay described above, is far from presenting an adequate description of the level of RNA synthesis *in vivo*. Large

discrepancies have been shown to exist between the rate of *in vivo* and *in vitro* synthesis although both determinations are fraught with their own inherent errors. The assay of RNA polymerase is, in general, based upon maximising the rate of incorporation of an isotope-labelled precursor into RNA. This can be achieved by magnesium concentrations in the region of 5mM, of manganese 2mM and of ammonium sulphate of 300mM. Estimates of magnesium concentration in the nucleus prepared by organic solvents suggests a high internal level, as compared to the cytoplasm, of 16mM (LANGENDORF et al, 1961) and 12mM (OKAZAKI, SHULL and FARBER, 1968) much greater than that found optimal in any *in vitro* assays. Manganese concentrations in the nucleus of rat liver have been estimated at 0.05mM (FORE and MORTON, 1952; THIERS and VALLEE, 1957). Ions are known not only to be concentrated in the nucleus but to be concentrated regionally within it, especially at sites of active RNA synthesis during meiosis (TRES et al, 1972).

The action of ammonium ions has been suggested to be either the inhibition of degradative enzymes (STEINER and KING, 1966), exposure of more DNA template by the removal of histones (CHAMBON, RAMUZ and DOLY, 1965) or the activation of latent enzyme molecules (WIDNELL and TATA, 1966). Whatever their action is which causes the altered activity of RNA polymerase, there seems to be little doubt that their effect is different on the different forms of the enzyme. Roeder and Rutter (1969), and more recently Banks and Johnson (1973), have demonstrated this very clearly, and some of the products of the reactions have been partially defined.

With the characteristics of the RNA polymerase in nuclei from one-day old chick brain defined and a standard assay developed (above) it was possible to look for changes in its specific activity in the brains of birds which had been exposed to an imprinting stimulus. It was foreseen that this might be a difficult investigation to perform because of the variations in enzyme activity between birds from the same hatch, which could conceivably mask any small changes in activity. In the first experiments therefore a pilot study was run using only one exposure time (30 minutes).

LEGENDS TO FIGURES 9-15.

Fig. 9 A Teflon holder for washing glass fibre filters from the RNA polymerase assay. The filters were placed in the central well, covered top and bottom by the perforated covers and immersed in the washing media. (Drawn to original size)

Fig. 10 RNA polymerase activity (pmol ATP incorporated/mg DNA) in chick brain nuclei at times up to 30 minutes of incubation at 37°C. The assay medium consisted of: Tris-HCl, 100mM (pH 8·5); $MgCl_2$, 5mM; $(NH_4)_2SO_4$; 300mM; β-mercaptoethanol, 5mM; CTP, GTP and UTP, 0·5mM; ATP, 0·1mM; 0·167 μCi of (8-^{14}C)-ATP. Values are means. (N = 7 at all points)

Fig. 11 RNA polymerase activity (pmol ATP incorporated/minute) in chick brain nuclei at various concentrations of enzyme (measured as DNA content). The incubation was 5minutes at 37°C. The assay medium was as for Fig. 10. Values are means. (N = 7 at all points)

Fig. 12 RNA polymerase activity (pmol ATP incorporated/mg DNA) in chick brain nuclei at several pHs. The incubation was for 5 minutes at 37°C. The assay medium was as for Fig. 10 except that the pH of the Tris-HCl was varied as necessary. Values are means. (N = 7 at all points)

Fig. 13 RNA polymerase activity (pmol ATP incorporated/mg DNA) in chick brain nuclei at several concentrations of magnesium. The incubation was for 5 minutes at 37°C. The assay medium consisted of: Tris-HCl (pH 8·5), 100 mM; β-mercaptoethanol, 5mM; CTP, GTP and UTP, 0·5mM; ATP, 0·1mM; 0·167 μCi of (8-^{14}C)-ATP. $MgCl_2$ was added at the appropriate concentrations. $(NH_4)_2SO_4$ was either omitted (open circles) or added at 300mM (closed circles). Values are means. (N = 7 at all points)

Fig. 14 RNA polymerase activity (pmol ATP incorporated/mg DNA) in chick brain nuclei at several concentrations of manganese. The incubation was for 5 minutes at 37°C. The assay medium was as for Fig. 13 except that $MnCl_2$ was added instead of $MgCl_2$. $(NH_4)_2SO_4$ waseither omitted (open circles) or added at a concentration of 300mM (closed circles). Values are means. (N = 7 at all points)

Fig. 15 RNA polymerase activity (pmol ATP incorporated/mg DNA) in chick brain nuclei at several concentrations of ammonium sulphate. The incubation was for 5 minutes at 37°C. The assay medium was as for Fig. 13 except that $(NH_4)_2SO_4$ was added at the appropriate concentrations. $MgCl_2$was added at a concentration of 5mM (closed circles) or $MnCl_2$ was added at 2mM (open circles). Values are means. (N = 7 at all points).

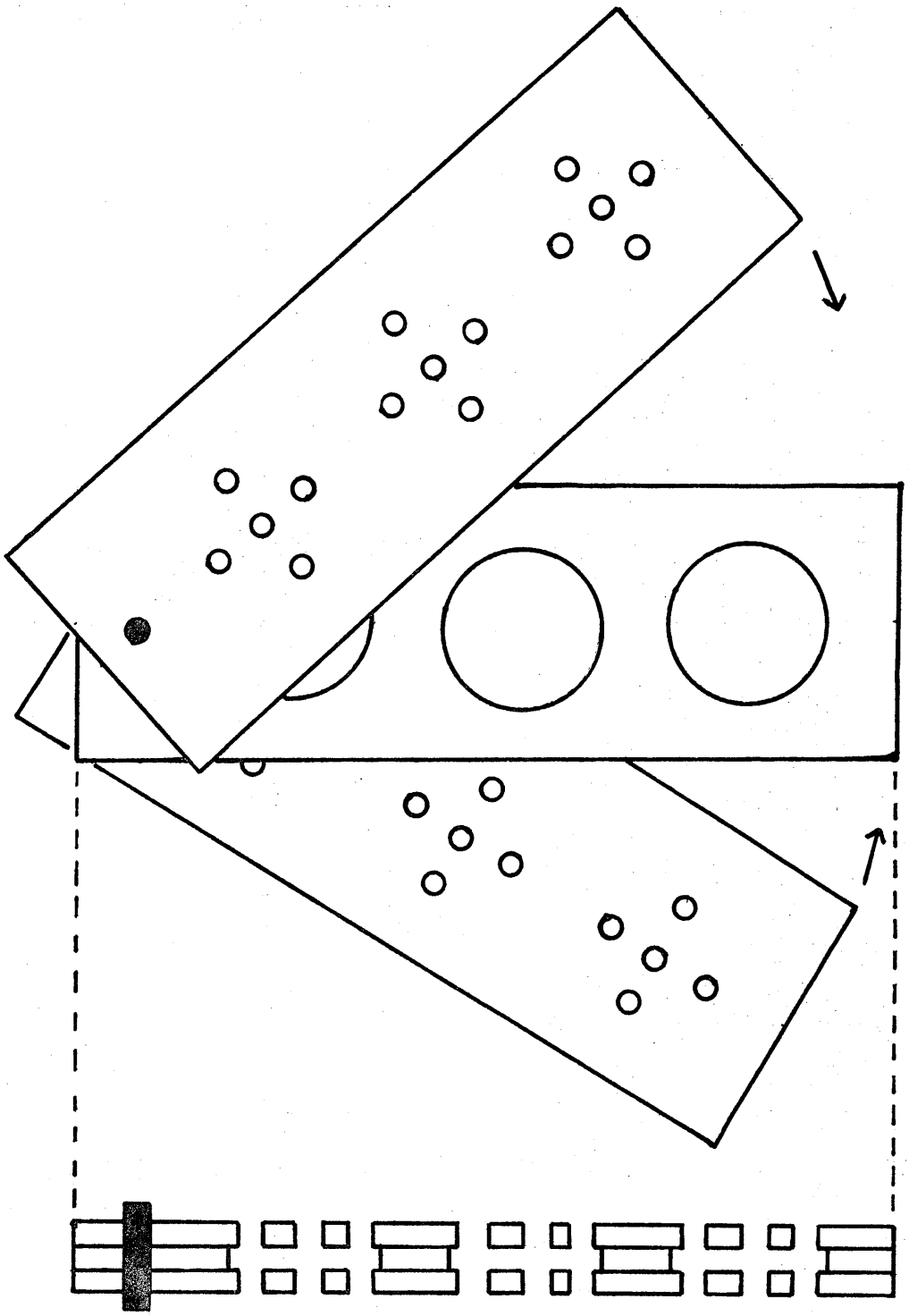


FIG. 9

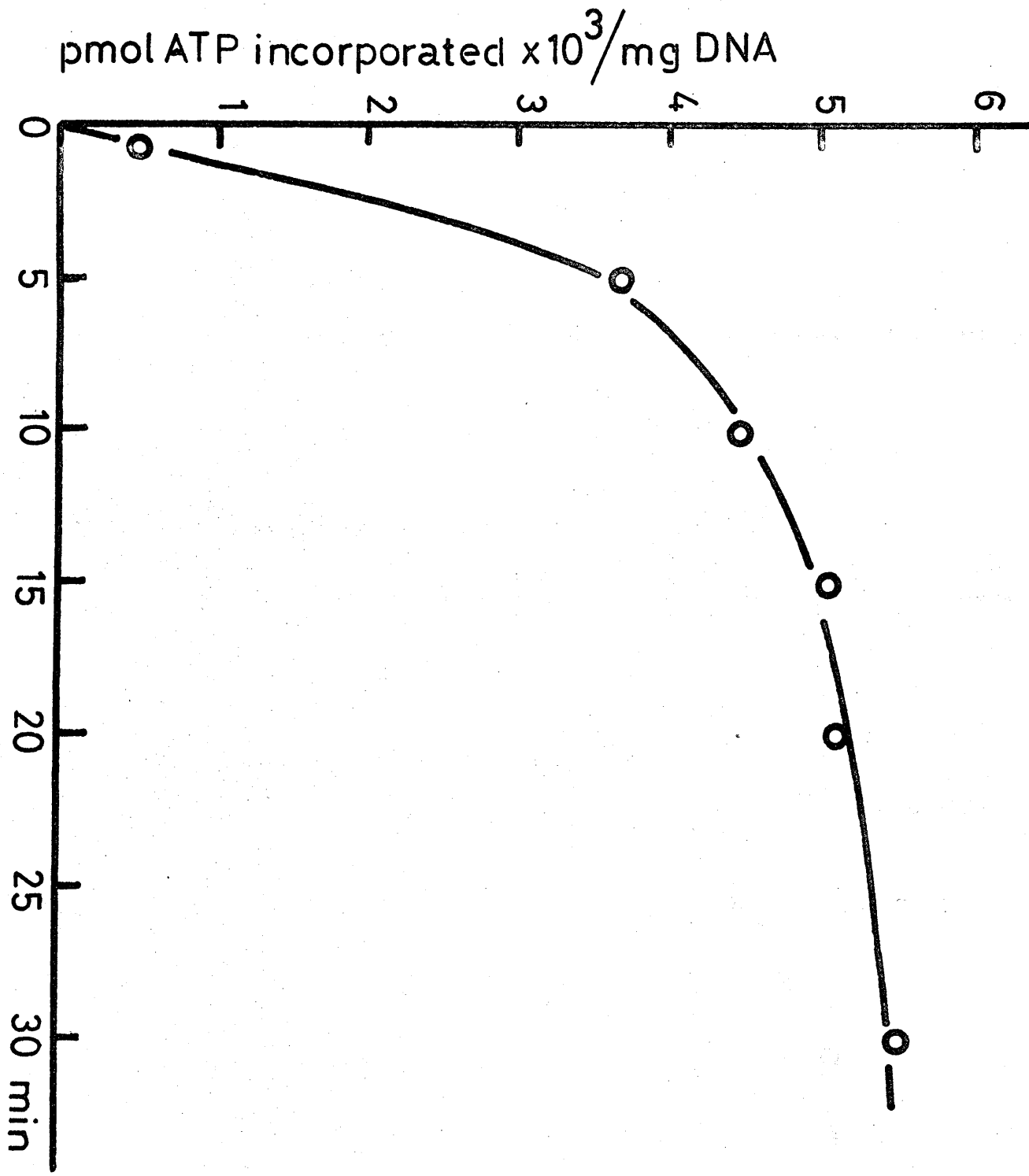


FIG. 10

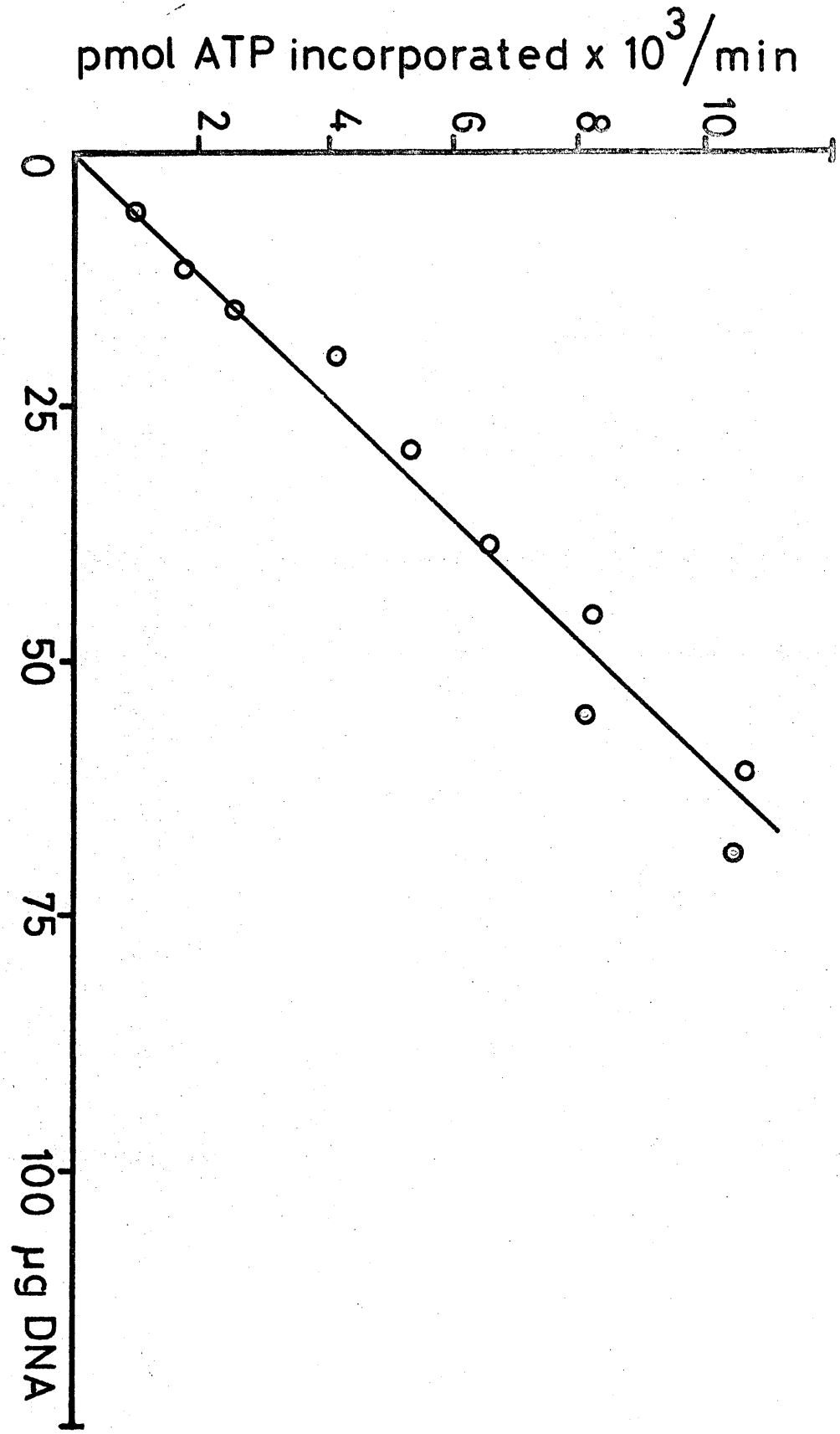


FIG. 11

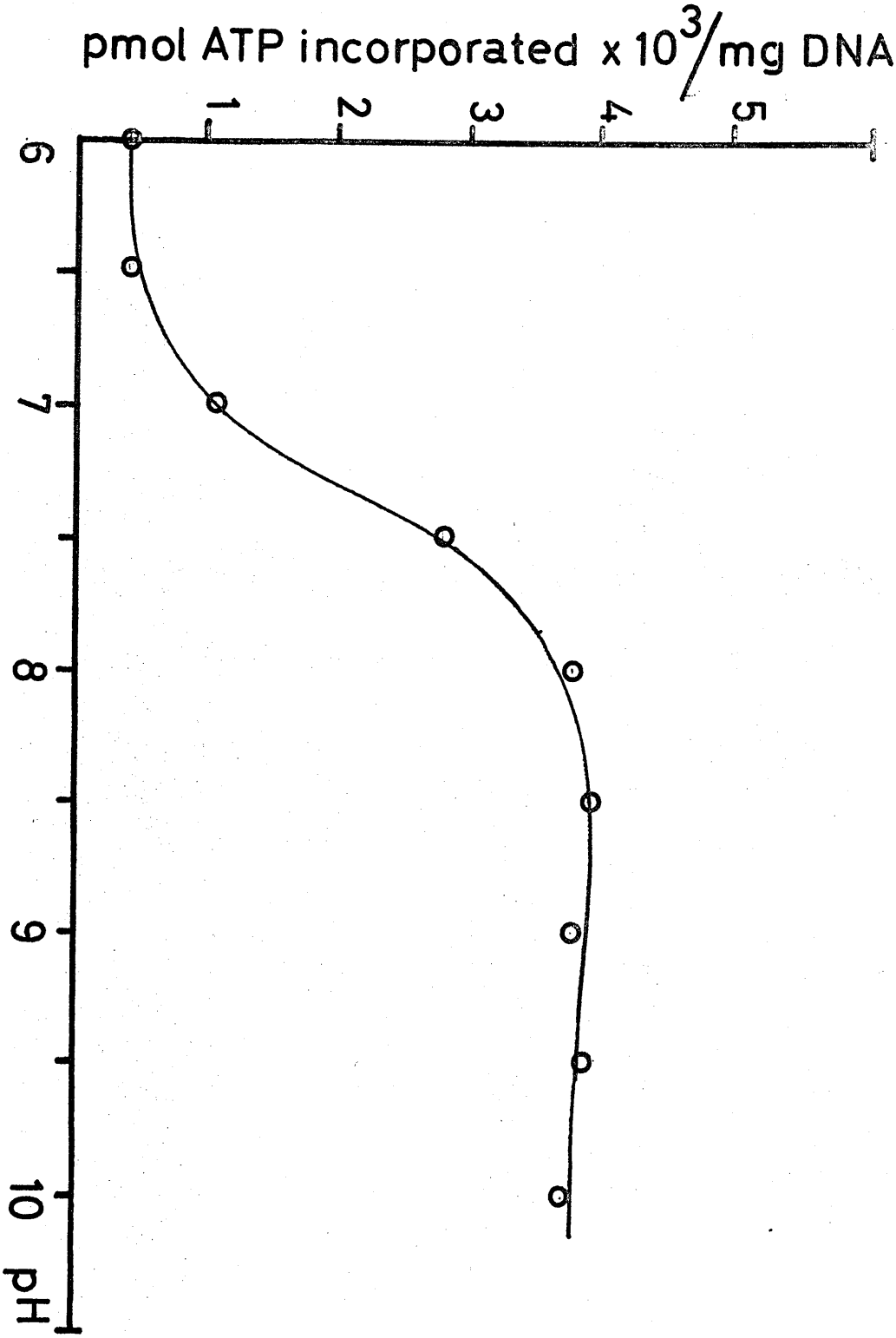


FIG. 12

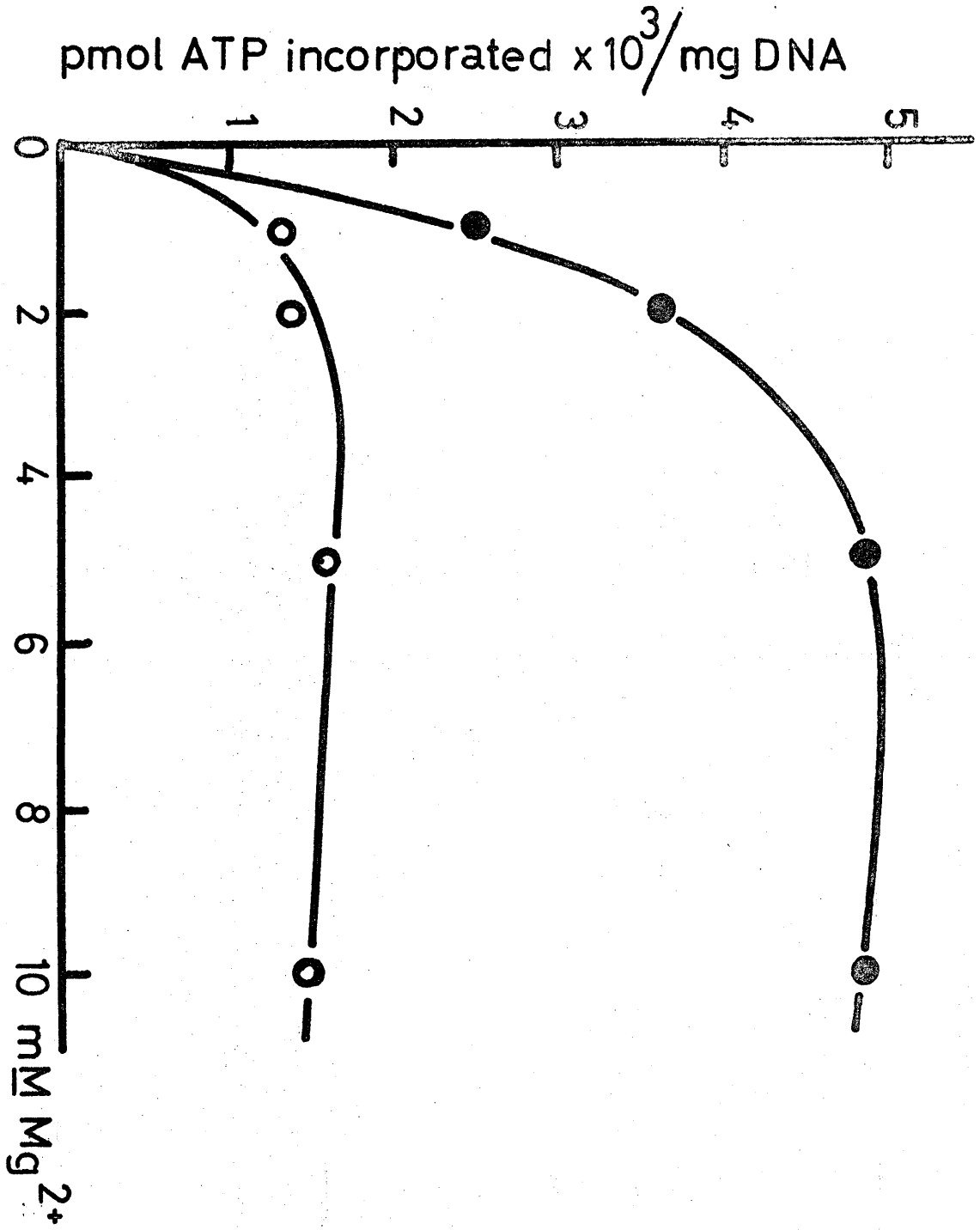


FIG. 13

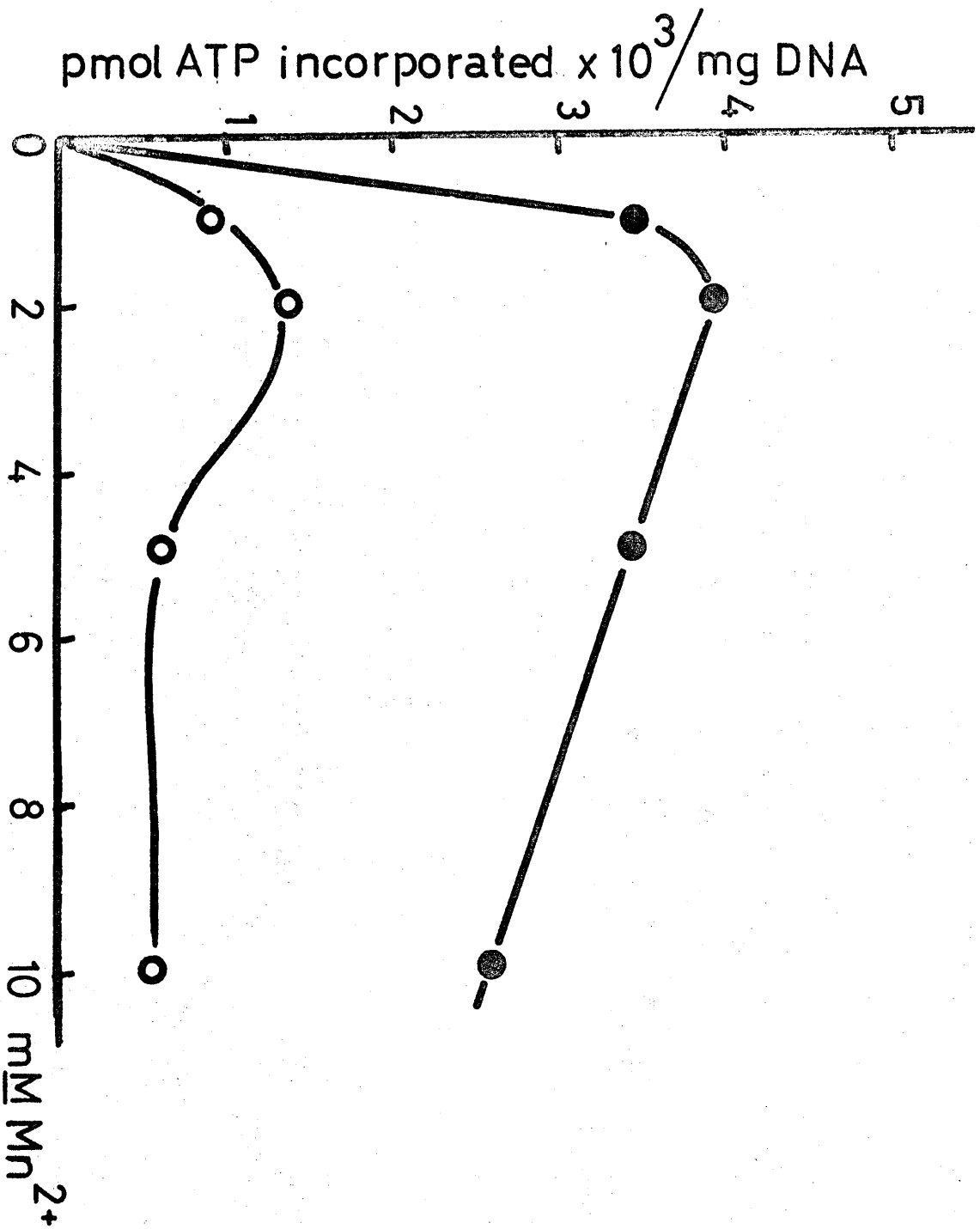


FIG. 14

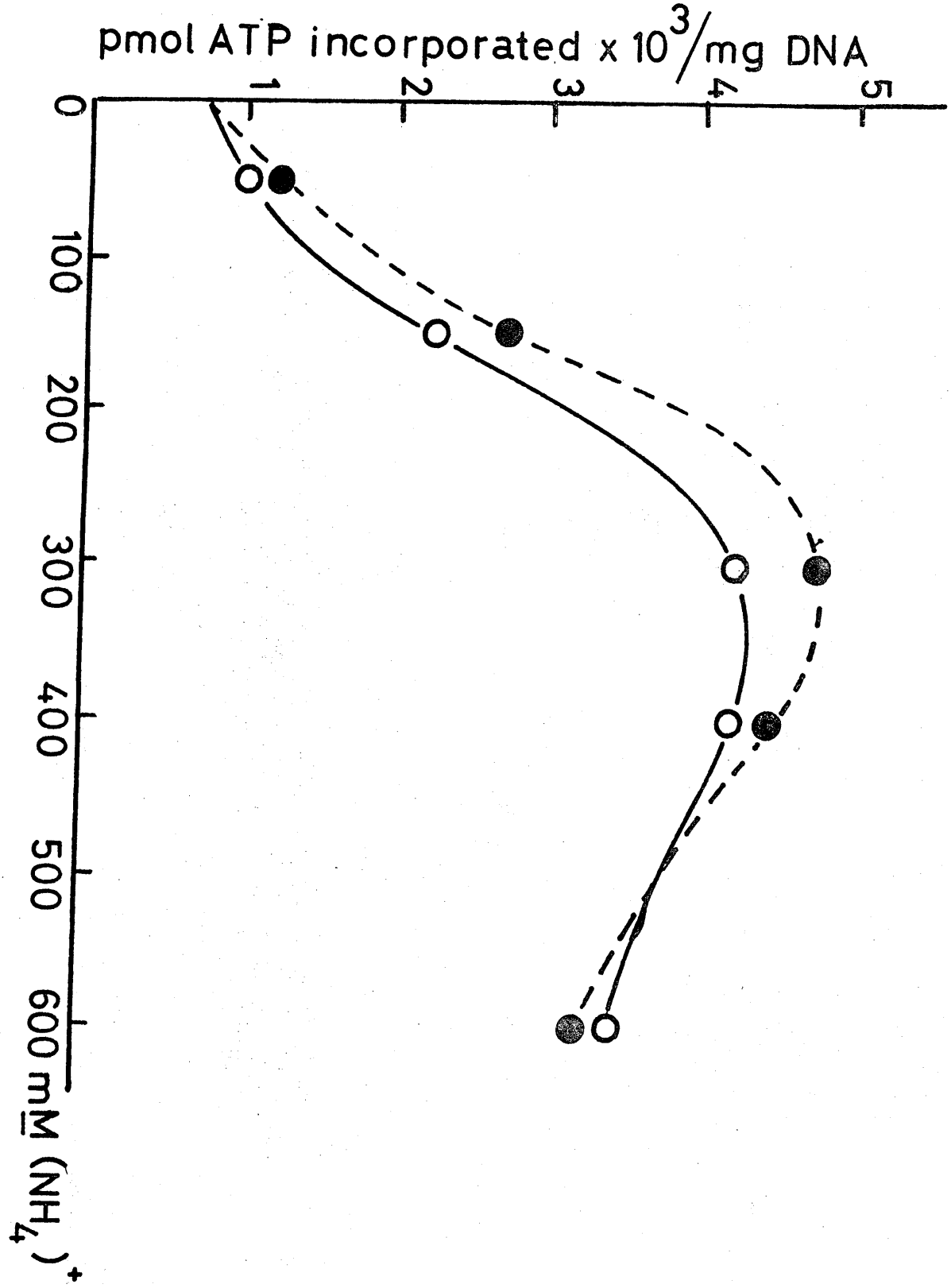

pmol ATP incorporated x 10³/mg DNA
1
2
3
4
5
0
100
200
300
400
500
600 mM (NH4)+

FIG.15

CHAPTER V

The results of the experiments described in Chapter II gave an outline of the changes which occur in brain protein and nucleic acid metabolism of chicks when they are exposed to an imprinting stimulus. The most detailed information came from the study of the effects on uracil incorporation into presumed RNA of different periods of exposure (BATESON et al, 1972). After 38 minutes' treatment there were no differences in precursor incorporation between the three types of bird, stimulus-exposed (E), diffuse light-exposed (L) and dark-maintained (D) but after 76 minutes the incorporation was higher in the E than both L and D birds, but in one brain region only, the forebrain roof. By 150 minutes' treatment both the E and L birds had higher uracil incorporation in all three brain regions than the D birds.

If these enhanced incorporations were the result of increased synthesis of RNA and not due to alterations in the pool specific radioactivity of the uracil it was possible that such an increase had been effected through increased RNA polymerase activity. (Such an increase in activity would not be mandatory, however, because increased supply of rate-limiting factors (e.g. ATP) could produce the same elevated RNA synthesis. It is unlikely that such an effect would be seen in the <u>in vitro</u> assay where the extra-nuclear conditions are identical for all the nuclei.) It was predicted that a delay would be found between the time of any change in RNA polymerase activity and the detection of alterations in the rate of RNA synthesis as measured by uracil incorporation. This is because although the change in enzyme activity could be

virtually instantaneous and would be detected as such, the detection of a differential in specific activities of RNA requires sufficient synthesis to occur to incorporate enough radioactive uracil for the absolute magnitude of the difference to exceed the error in the measurement. Just such a delay time was observed in the reaction of nucleic acid and protein metabolism to hormones (HAMILTON et al, 1968). Thus as the exposure time points for measurement of uracil incorporation had been 38, 76 and 150 minutes, and as no difference in incorporation was detected before 76 minutes, it seemed reasonable to choose times between 0 and 76 minutes. For the preliminary experiment, to see if any change at all was detectable, a time of 30 minutes was chosen. To reduce the experiment to the simplest possible design, only stimulus-exposed and dark-maintained birds were used, the diffuse light-exposed birds being omitted.

The birds were all hatched and treated in equipment at the Sub-Department of Animal Behaviour, Department of Anatomy, The University of Cambridge, at Madingley, Cambs., according to the methods developed there by P.P.G. Bateson. This was the same equipment in which the earlier uracil experiments had been performed. The frozen tissue samples were then brought to the Open University to be assayed for nuclear RNA polymerase activity.

Materials and Methods:

Animals Ross I type (commercial broiler strain) chicks were used throughout these studies. Fertile, unincubated eggs were obtained from Ross Poultry Products, Little Downhan, Cambs., and incubated for 18 days at 37.6 $\pm$ 0.1°C in a Western moving-air incubator which had been made light-tight. At the end of

this time they were transferred in darkness, to a Curfew observation incubator at 40°C. The birds hatched in darkness on approximately Day 21 after the beginning of incubation and were transferred, again in darkness, to a blacked-out brooder incubator at 33.5 ± 0.5°C, generally within 2 hours of hatching. The time required for all the birds to hatch (on average 85% of those incubated) was approximately 24 hours, the time at which half the birds had hatched being called the 'hatch midpoint'. Only those birds which hatched in the 9-hour period prior to the midpoint ('early hatchers') were used in imprinting experiments. This is the same criterion as that used by Bateson (BATESON et al, 1969). The birds were used for experiments at a mean age of 18 ± 0.5 hours post-hatch. Six birds were drawn from each hatch; three were exposed to the stimulus and three were kept in the dark.

Chemicals All the chemicals used were the same as those in Chapters III and IV.

Behaviour The stimulus to which the birds were exposed consisted of a Perspex box (18cm wide x 9cm deep x 18cm high), the larger sides being opal and the smaller sides and top black. The box was mounted on an Eisenmann recovery vehicle hazard lamp base and rotated around the central 48W bulb at 85 rev/minute (Fig 16). Between the bulb and the walls of the box was a cylinder of orange celluloid (Cinemoid No. 5; Strand Electric, London) so that the effect seen, when viewed from the side was one of a flashing, orange light. The stimulus was situated at the centre of a circle of, and 45cm from, 12 pens in which the chicks were placed (Fig 17), which were approximately 30cm in each direction and painted matt battleship grey. Six of the pens had fronts (that

is facing the stimulus) made of hardboard (L pens), and the other six had fronts of ½" weld mesh (E pens) so that the birds could see, but not approach, the stimulus. The roof of the centre of the arena, that is the area from the fronts of the pens inwards, was covered over to prevent light from the 40W pearl bulb, at 2 metres above the centre of the arena, from reaching the E pens through the front. Thus all the pens received diffuse overhead illumination but only the E pens received light from the stimulus. The dark-maintained birds (D) were kept in a dark-box, with 9 pens (30cm x 30cm x 30cm) covered by a light-proof cloth, next to the arena. Thus the D birds experienced the same sound and temperature sensations as the E and L birds. The temperature in all the apparatus was 30 $\pm$ 0.2°C, and was maintained by thermostatted circulating air from two 3kW industrial fan heaters. Frequent checks were made to ensure that the temperature did not vary from these limits.

This temperature is several degress below that preferred by one-day old chicks (HERBERT and SLUCKIN, 1969) but ensures that they do not fall asleep during the experiment (BATESON et al, 1972).

The chicks were tested at the end of the experiment, immediately prior to killing, in a matt-grey alley 120cm long x 45cm wide x 30cm high (Fig 18). One end of the alley was blank and the other was sealed off with ½" weld mesh, 30cm behind which was a stimulus identical to that described above. Each chick was tested singly. It was placed in darkness with its beak pointing towards the side wall of the alley, and its movements were recorded from the moment at which the stimulus was switched on. It was observed by means of a mirror, which was the same length as the alley, placed over the alley at an angle of 45°. Thus the

observer, who was in a darkened part of the room, could see the bird but it was unlikely to see him and be distracted. The temperature in the alley was 26.0 $\pm$ 0.5°C. The time taken for each bird to reach a line 15cm from the stimulus-end of the alley was recorded if this was less than the test time of 120 seconds. All birds were left in the alley for this length of time even if they had reached the mesh much earlier. At the end of its test each bird was removed from the alley and killed by decapitation. The brain was removed and dissected rapidly into three regions: forebrain roof, forebrain base and midbrain (see p.21). The cerebellum was discarded. The tissue portions were frozen in coded, numbered pots on solid CO_2 and stored at -20°C until being assayed for nuclear RNA polymerase.

Preparation of nuclei and assay of RNA polymerase activity

This was done according to the methods described in Chapters III and IV.

The six samples from one stimulus-exposed and one dark-maintained bird were always worked up and assayed simultaneously, but the identity of each member of the pair was unknown at the time. This code was not broken until all the samples from an experiment had been processed.

Results

Behavioural To determine whether or not a bird has been imprinted on a stimulus requires the demonstration that it will choose that stimulus when presented with it in the presence of another, originally equally effective, stimulus. The presentation of the stimulus alone, with the bird in slightly stressful conditions (as here),

gives an indication of its degree of arousal or activation but this cannot be taken as a true measure of the extent of imprinting as similar results can be obtained by merely exposing the birds to plain light. However, in spite of the limitations of these measurements, it was felt that it was worthwhile making these observations to check that, at the least, arousal had occurred. If this was so it could be expected that the birds had indeed developed some degree of imprinted behaviour by reference to earlier behavioural studies (BATESON and REESE, 1969).

Table 11 shows the effect of 30 minutes' treatment on the approach times of the two types of birds, stimulus-exposed (E) and dark-maintained (D). The values given are the median times for each group.

TABLE 11

Approach behaviour of chicks after 30 minutes' treatment

Bird type	Median approach time (sec)
Stimulus-exposed (E)	68
Dark-maintained (D)	>120

Median approach times (seconds) to within 45cm of the stimulus in a test-alley for chicks exposed to 30 minutes of stimulus or darkness. ($N = 17$; $U = 55$; $P \leq .002$)

It was not possible to calculate the mean approach times because not all birds reached criterion, that is to within 15cm of the stimulus-end of the alley within the test time of 120 seconds.

This was particularly true for the dark-maintained birds. For this same reason the data was analysed for the level of significance of the difference between the medians by the Mann-Whitney U test. As had been found in previous experiments (BATESON et al, 1972) the stimulus-exposed birds approached the stimulus much more rapidly than did the dark-maintained birds, many of which did not move from their starting position. Thus in terms of their behaviour the birds used in these experiments were comparable to those which had been used in the precursor incorporation experiments.

RNA polymerase activity

As discussed in Chapter IV, there were variations in the mean enzyme activity between different hatches of birds and consequently the data had to be normalised to minimise the scatter. Instead of normalising against a chosen activity value, e.g. 4000 pmol ATP incorporated/mg DNA/5 minutes, an internal standard was used from each experiment. All the samples from the birds had been worked up and assayed as E-D pairs and so these should be internally consistent as to the effects of the nuclear preparation and assay on the enzyme activity. The enzyme activity data, in disintegrations per minute (dpm) incorporated/mg DNA, from each brain region of the two animals of the E-D pair were expressed as percentages of the mean activity of all of them. Thus for the forebrain roof (fr) of the stimulus-exposed bird (E), the percentage relative activity was $\frac{A_{r-E}}{\bar{A}_{E-D}} \times 100\%$ where $\bar{A}_{E-D} = (A_{r-E} + A_{b-E} + A_{m-E} + A_{r-D} + A_{b-D} + A_{r-D} + A_{b-D} + A_{m-D})/6$

This data is shown in Table 12.

TABLE 12

Relative RNA polymerase activities in nuclei from three brain regions of chicks exposed to an imprinting stimulus for 30 minutes or kept in darkness

Bird type	Brain Region		
	Forebrain roof (%)	Forebrain base (%)	Midbrain (%)
Stimulus-exposed (E)	110.3 ± 7.6	124.3 ± 8.6	109.6 ± 8.6
Dark-maintained (D)	82.4 ± 7.9	102.4 ± 9.3	97.6 ± 7.3
E/D	1.34	1.21	1.12
P ≤	0.02	0.09	N.S.

The results are calculated as percentages of the mean activity of all the brain regions of an E-D pair (see text). Values are mean ± s.e.m. Significances are calculated by Students' t-test. (N = 17, except roof of E, where N = 15)

There was a significant increase ($P \leq 0.02$) of 34% in RNA polymerase activity in the forebrain roof of stimulus-exposed birds as compared to dark-maintained, but no significant differences in the forebrain base or midbrain.

This substantial elevation in enzyme activity in the forebrain roof after 30 minutes of exposure to the stimulus was greater than had originally been expected. In the ^{3}H-uracil incorporation studies a 17% increase had been found in incorporation in the forebrain roof only of 76-minute stimulus-exposed as compared to dark-maintained birds. This was after 150 minutes of incorporation, and this increase had probably only occurred within the last 75 minutes of labelling time (see Fig 19). No difference between the 76-minute E and the D birds would have

existed in the first 37 minutes of incorporation in darkness because their conditions had been identical, and during the next 38 minutes of exposure no difference in incorporation had existed such as to have caused any detectable differential after the 150 minutes incorporation, at least in the 38-minute treated birds. Thus all incorporation changes had occurred within the next 150 - (37 + 38) = 75 minutes. The 17% difference found could have been due to either or both of increased synthesis and reduced degradation of RNA (assuming that the net synthesis rate of RNA had altered and the effect was not due to pool specific radioactivity). If no degradative changes had occurred, a maximum increase in RNA synthesis of 17% would have had to have occurred to have produced the +17% incorporation difference at 150 minutes, and hence a constant 17% increase in enzyme activity over that period would have been needed to produce it (Fig 19). It seems unlikely that such a simple model could hold true as an explanation of these events. A shorter period of increased RNA polymerase e.g. +34%, over about half the 75-minute period, would have produced the same result; in other words the increased enzyme activity may have lasted only as long as the stimulus was present, there being no difference in uracil incorporation rates over the second dark period. Also the delay time between the onset of increased activity of RNA polymerase and the detection of an increase in its product, RNA, measured by uracil incorporation is unknown.

This preliminary study demonstrated the possibility of measuring changes in RNA polymerase activity in cerebral nuclei in response to altered environmental stimuli, the first time that this had been done. It is suggested that the effects of

increased ^{3}H-uracil incorporation reported previously had some basis in altered RNA synthesis because the *in vitro* enzyme assay, although not a true measure of RNA synthesis *in vivo*, was free from the problems of changed precursor pool specific radioactivity.

However the difference in magnitude of the two aspects of the response of cerebral RNA metabolism to the stimulus indicated that a very simple model of their interrelationship was not valid and it was therefore of interest to look at the activity of RNA polymerase at both shorter and longer times of exposure to try to clarify this point.

(The results upon which this chapter is based have been published - HAYWOOD et al, 1970.)

LEGENDS TO FIGURES 16-19.

Fig. 16 Stimulus to which birds to be imprinted were exposed. The two shorter sides of the Perspex box were black, the longer sides were opal. The box rotated at 85 rev/min around the central bulb, which was enclosed in an orange filter. (b=bulb; o=filter)

Fig. 17 The equipment in which the stimulus- and diffuse light-exposed birds were placed during the experiment ("arena"). The E pen fronts were made from weld-mesh (dotted lines) and those of the L pens from hard-board (solid lines). The stimulus was placed at the centre of the arena. The temperature in the apparatus was 30°C.

Fig.18 The equipment in which all birds were tested for their response to the stimulus after treatment ("alley"). Birds were placed on the central circle and were free to approach the stimulus (LHS of diagram) or withdraw from it. The temperature in the alley was 26°C.

Fig. 19 Possible changes in RNA polymerase activity which could account for the 17% in crease in ^{14}C-uracil incorporation in 76-minute stimulus-exposed birds.

U = extent of uracil incorporation

P = RNA polymerase activity

E = stimulus-exposed birds

D = dark-maintained birds

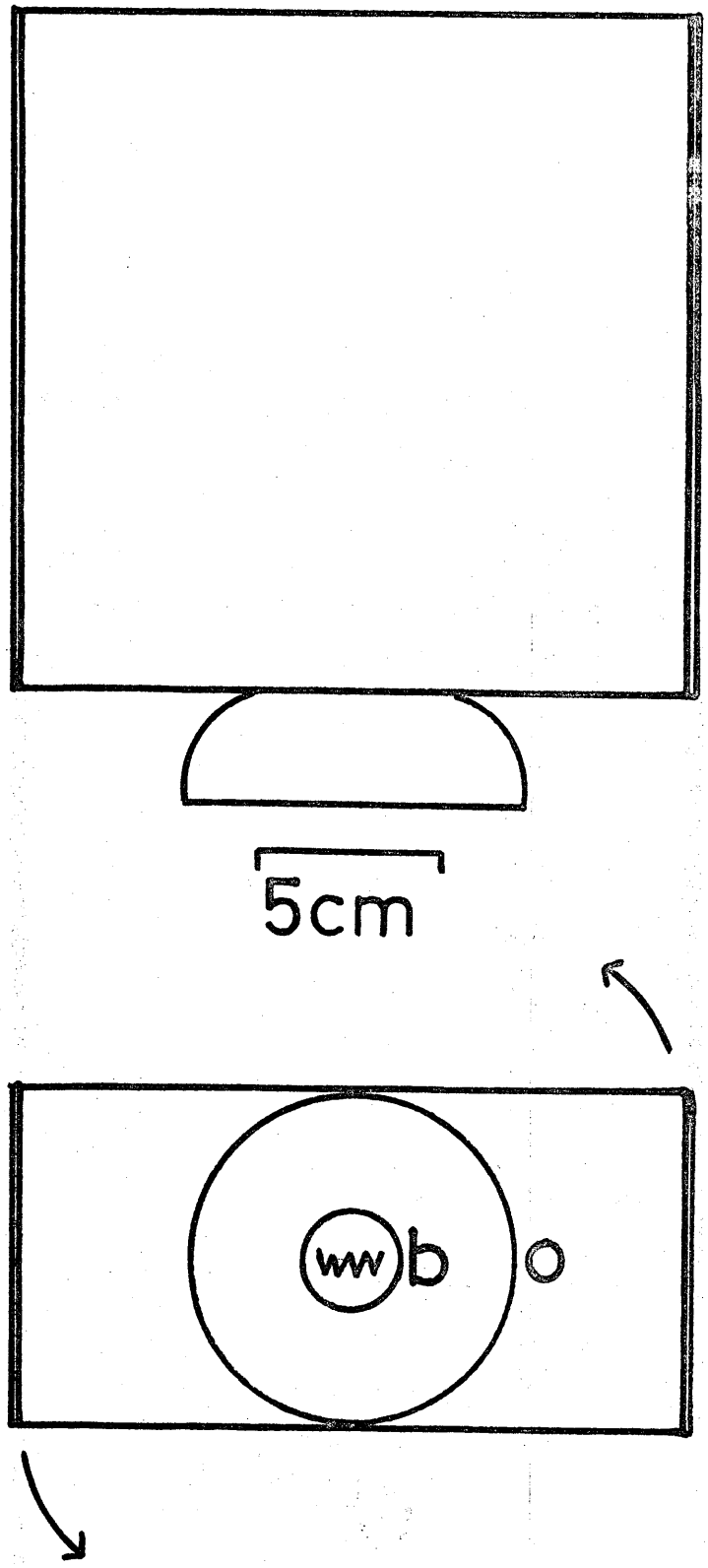


FIG.16

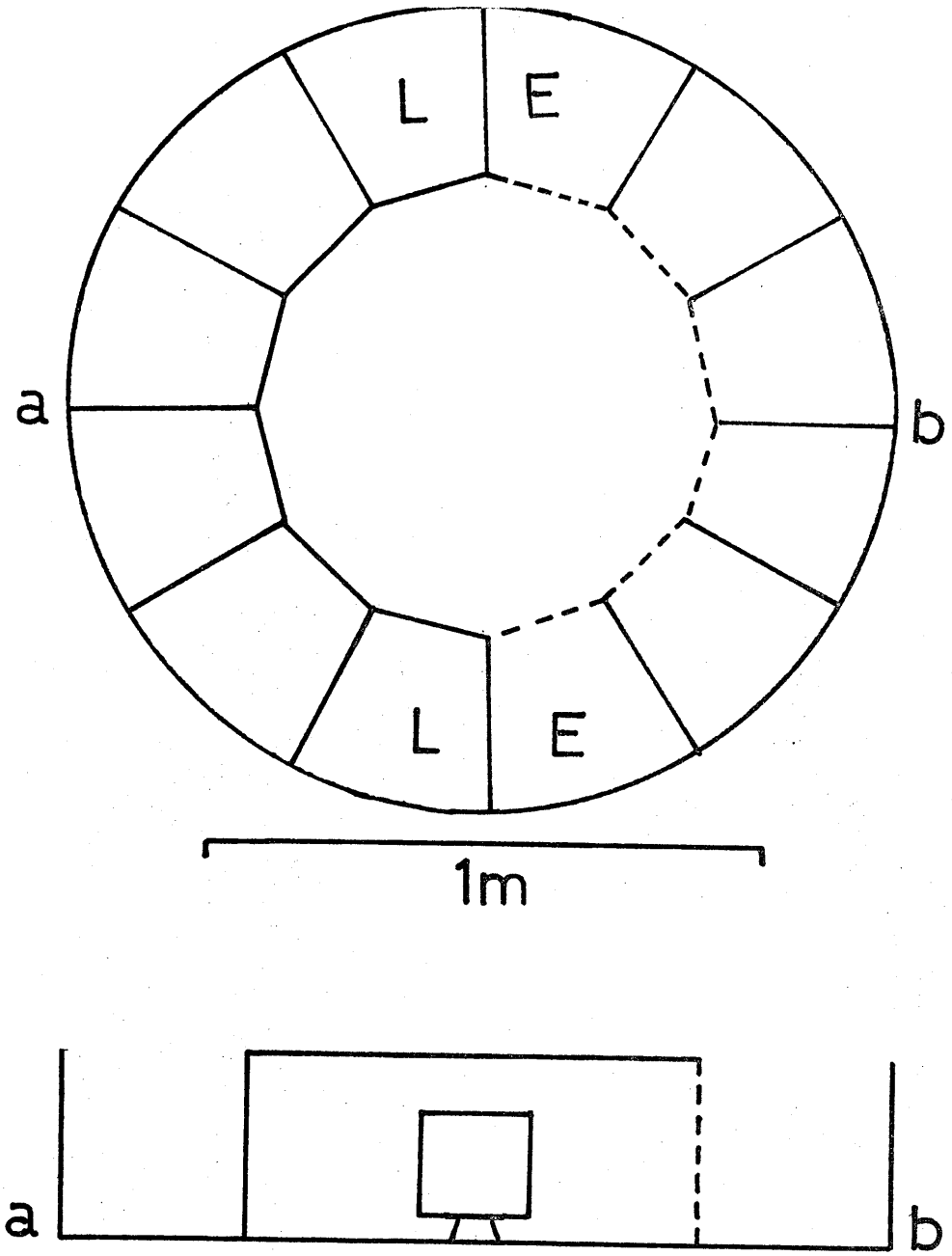


FIG.17

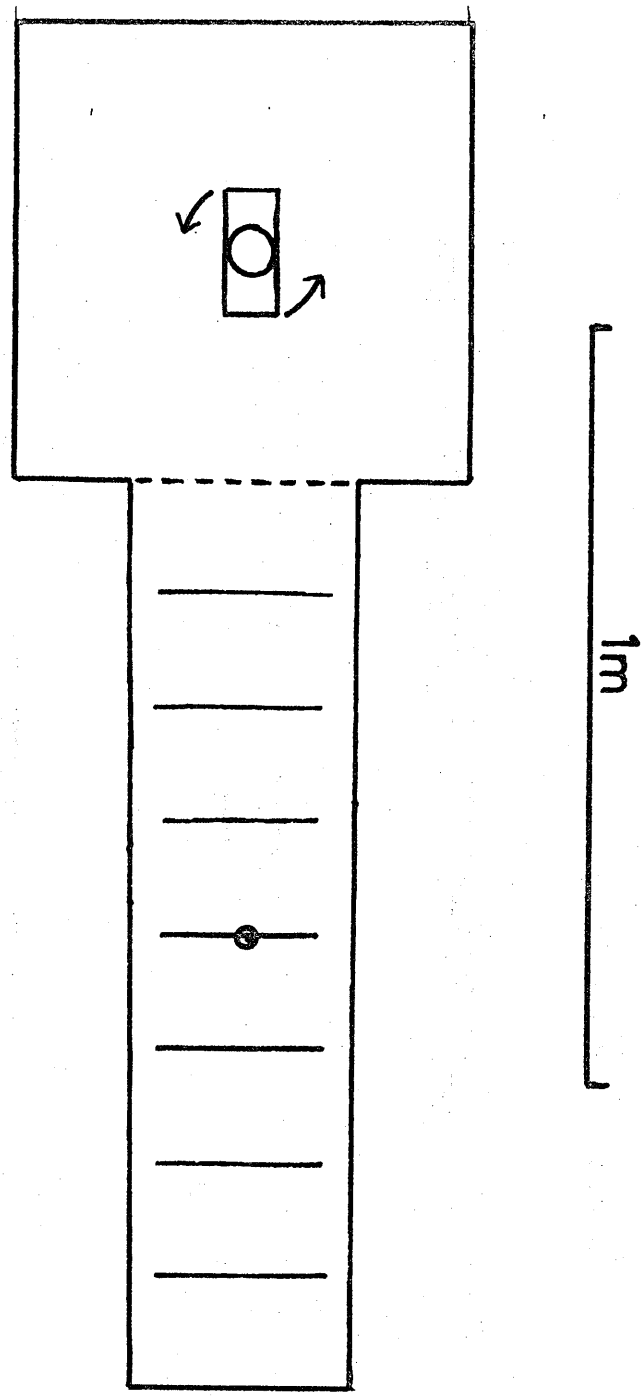


FIG.18

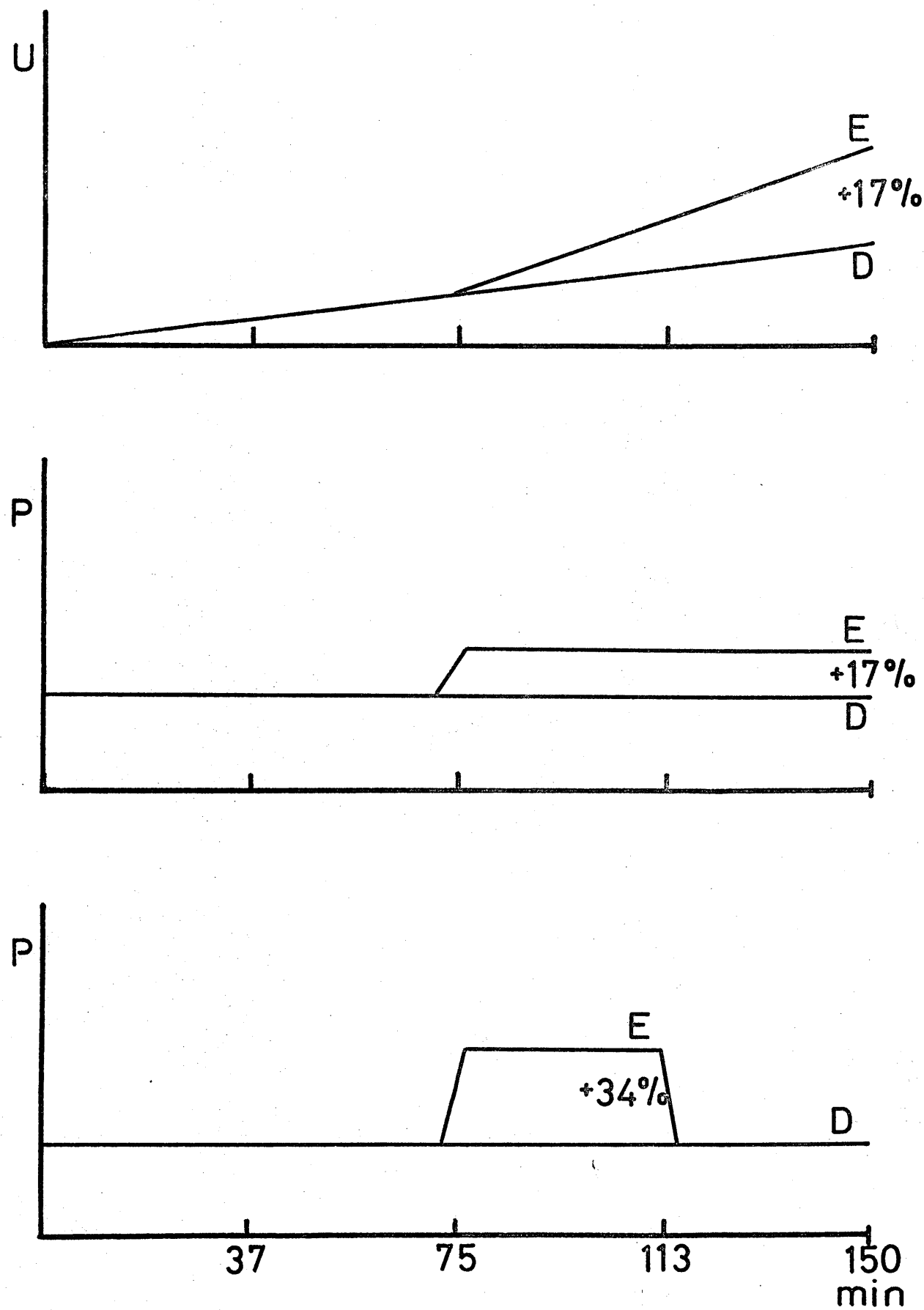


FIG. 19

CHAPTER VI

The decision to extend the scale of biochemical operations on imprinted chicks necessitated the construction of behavioural training equipment at Milton Keynes. This was identical to that sited at Madingley and as described in Chapter V. In order to check that the results obtained at Madingley in previous experiments were reproduceable, and thus that the results of further experiments would be compatible and comparable with them, an experiment originally done at Madingley was repeated at Milton Keynes. The experiment chosen was the study of the effects of different periods of exposure of chicks to the imprinting stimulus on the incorporation of uracil into brain RNA. This was chosen because it gave the maximum amount of information about the state of the birds, both behavioural and biochemical, of all the experiments so far performed, yet required relatively simple biochemical manipulations. Although there were several points open to criticism in the experimental design (see p.24) the same schedule of events was followed so that a direct comparison could be made with the earlier results. However the use of tritium as isotope was felt to be too likely to introduce errors into the determination of the extent of uracil incorporation (see p.7)and consequently ^{14}C-uracil was used instead.

Materials and Methods:

Animals Ross I chicks were hatched as described in Chapter V and used at a mean age of 18 hours post-hatch.

Chemicals Uracil-2-14C (specific activity >50 mCi/mmol) and uracil-5-3H (specific activity 1000 mCi/mmol) were from the Radiochemical Centre, Amersham, Bucks., U.K.

NCS solubiliser was from Amersham/Searle, Illinois, U.S.A.

PPO scintillator (2,5-diphenyloxazole) was from Packard Instrument Co., Illinois, U.S.A.

Bovine serum albumin and uracil was from Sigma Chemical Co., London, U.K.

All other reagents were from BDH, Poole, Dorset, U.K., and were of Analar Grade.

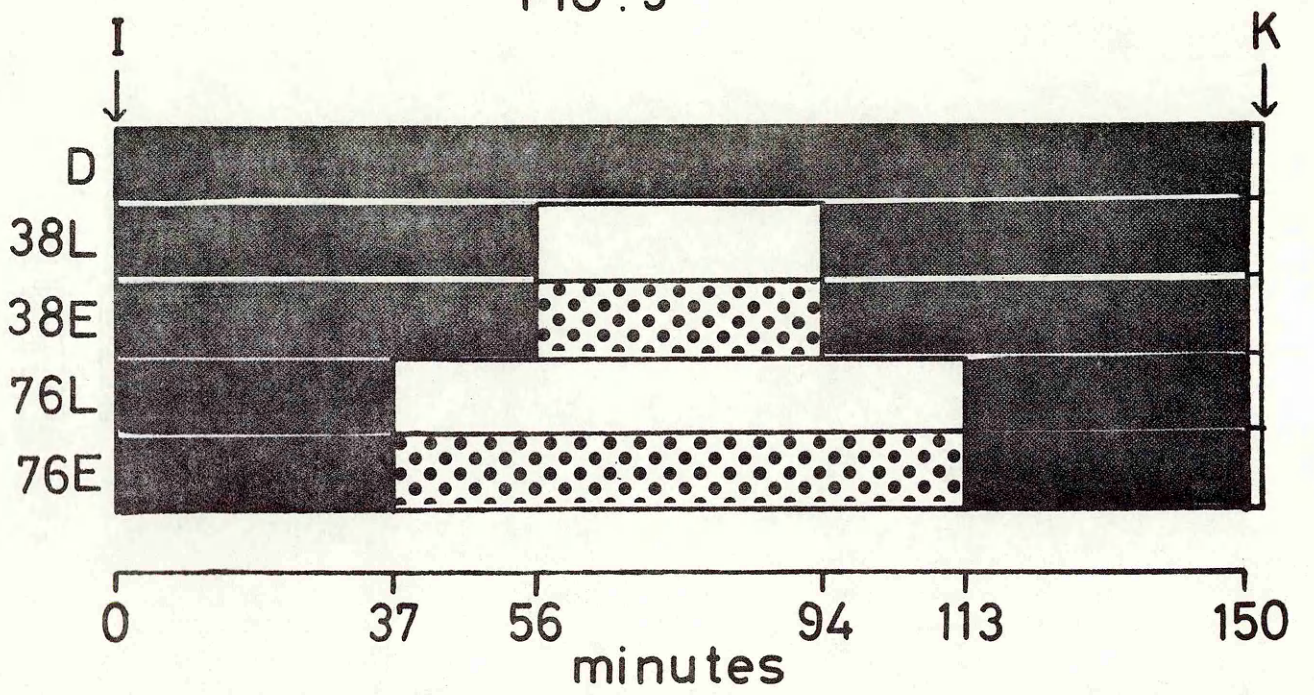
Behaviour and Biochemistry:

Ten birds were taken from each hatch and divided into five equal groups:

dark-maintained	(D)
38-minute diffuse light-exposed	(38L)
38-minute stimulus-exposed	(38E)
76-minute diffuse light-exposed	(76L)
76-minute stimulus-exposed	(76E)

Schedules were drawn up so that the order in which birds were removed from the 33°C brooder incubator, exposed and killed was varied for each experiment to eliminate 'order-effects'. Each bird was labelled by means of a numbered, small self-adhesive label on its back. This code was varied so that when the samples were worked up and analysed (two days after the behavioural part of the experiment) each sample was to all intents and purposes unknown and an element of 'blindness' was introduced. The general design of the experiment was as in Fig 5.

FIG. 5



Immediately before injection each bird was numbered and weighed. The pericardiac injection was performed by inserting the full length of a 27 gauge needle (length 10mm) on a 1ml disposable syringe just below the centre of the caudal tip of the sternum at 45° to the vertical. Pilot experiments with dye showed that the tip of the needle missed the heart but was positioned close to it. 4μCi of ^{14}C-uracil were injected in a volume of 0.1ml of 0.9% (w/v) sodium chloride solution at 30°C.

Most birds seemed to suffer no obvious ill-effects after injection and very few died as a result of it.

Before repeating the experiments to observe the effects on uracil incorporation into chicks of exposure to the imprinting stimulus the basic kinetics of uracil uptake and incorporation were studied. This was done by injecting several 18 hour-old chicks as described above, keeping them at 30°C in the dark, and killing them after varying times. The birds were killed by decapitation, the skull opened and the forebrain, midbrain and cerebellum lifted out on a spatula. The cerebellum was cut away and discarded. The dissection was as described above (p. 21); the forebrain roof and base were separated with the aid of a plastic mould. This was made from black epoxy resin which had been allowed to set around a formaldehyde-fixed chick forebrain. The forebrain to be dissected was laid in it with its roof inside the moulded area and held lightly in position with one finger. A razor blade was then passed across the flat surface of the mould thus dissecting the (upper) base from the (lower) roof. On the basis of the wet weight ratio between roof and base, the error in dissection was in general only $\pm$ 10%. The same mould was used for all experiments to ensure the comparability of dissected tissue portions. These tissue portions were frozen in numbered pots on solid carbon dioxide and kept at -20°C until analysis. All the samples were worked up together. They were homogenized in 5.0 ml of 0.9% NaCl with an M.S.E. top drive macerator for 60 seconds at 2500 rpm. Aliquots were taken for protein estimation (LOWRY et al, 1951), total radioactivity and acid insoluble radioactivity. Total radioactivity (T) samples were placed

directly into scintillation vials containing 0.5ml NCS and heated at 40°C for 30 minutes to digest them. The acid-insoluble (AI) samples, on ice, had ice-cold 10% (w/v) TCA with 10mM uracil added to a final TCA concentration of 5%, were left for 30 minutes at 4°C to coagulate the precipitate and then centrifuged at 2000 gav for 15 minutes in an MSE Mistral centrifuge at 4°C. The supernatants were decanted, the pellets resuspended in 5% TCA with 10mM uracil and centrifuged as before. This was repeated two more times. The pellets were drained of excess liquid, had 0.5ml NCS added and were digested at 40°C in a heating block (Dri-Block, Techni Instruments, Cambridge). All the AI and T digested samples were dissolved in 10ml of a scintillant consisting of 6g/l PPO in a 1:1 mixture of toluene and methoxyethanol and counted for radioactivity in a Beckman LS150 liquid scintillation spectrometer. The results were expressed as acid-insoluble disintegrations per minute per mg protein (AI dpm/mg protein) or as acid-soluble disintegrations per minute per mg protein (which were calculated by (total - AI dpm) (AS dpm/mg protein). Thus the AI data represented the degree to which radioactive uracil had been incorporated into RNA and the AS data the amount of unincorporated radioactive uracil left in the tissue. All the data was related to the protein content of the tissue and not to its RNA and therefore there are inherent assumptions that, (a) the ratio of RNA to protein in all the tissues did not change over the course of the experiment, and that, (b) if the chick brains differed with respect to this ratio at the start of the experiment, such variations were randomly distributed throughout the several

experimental types of bird. It would have been more rigorous to have related the uracil incorporation to the tissue RNA content, but as the assay for RNA is considerably more difficult than that for protein this was felt to be unnecessary. It is rather unlikely that there were changes in the RNA : protein ratio over the experimental period of 150 minutes which could have been detected by standard whole tissue assays.

Results

Behaviour As before (Chapter V) the times taken for the birds to approach to within 15cm of the stimulus-end of the alley were recorded. The medians of these times are shown in Table 13.

TABLE 13

Median approach times (seconds) for each category of bird

Condition	Duration of exposure to condition (minutes) 0	38	76
Stimulus-exposed (E)	-	42	37
Light-exposed (L)	-	88	53
Dark-maintained (D)	>120	-	-

Median approach times (seconds) for groups of chicks exposed to darkness (D) and 38 or 76 minutes of stimulus (E) or diffuse light (L). ($N = 12$) The difference in the medians between the D group and all other groups is significant ($U \leq 19$, $P \leq 0.001$). The 38-minute groups are significantly different from each other ($U \leq 25$, $P \leq 0.005$) as are the 76-minute groups ($U \leq 39$, $P \leq 0.05$).

As expected from previous reports there was a significant decrease in the median approach times of the E and L groups with respect to the D group, and also differences between the E and L groups themselves after 38 and 76 minutes of treatment.

Biochemistry The rate of incorporation of ^{14}C-uracil into acid-insoluble material (presumed RNA) was approximately linear in all three brain regions for 2.5 hours after injection (Fig 20), and then levelled off rapidly. The amount of acid-soluble radioactivity, that is mostly free radioactive uracil, rose from its initial value at 0 minutes after injection to a peak (probably before 1 hour, the earliest time point on the graph) and then declined linearly over this same period (Fig 21). No differences in specific activity (as dpm/mg protein) were detected between the brain regions with respect to either incorporated or pool uracil (Table 14).

TABLE 14

Specific radioactivity of incorporated and free 14C-uracil in three brain regions of chick at several times after injection

	Brain Region			
Time (hr)	Roof	Base	Midbrain	Form
1	15.4 ± 2.4	13.9 ± 1.9	13.6 ± 2.2	AI
	255 ± 48	287 ± 29	241 ± 34	AS
2	26.6 ± 4.1	26.0 ± 3.8	24.9 ± 3.7	AI
	144 ± 27	151 ± 26	152 ± 31	AS
3	31.9 ± 5.5	30.0 ± 4.8	32.7 ± 6.6	AI
	78 ± 14	83 ± 18	81 ± 17	AS

Specific activities (dpm/mg protein) of ^{14}C-uracil in acid-soluble (AS) and acid-insoluble (AI) fractions of three regions of chick brain. Determinations were as in Methods. Values are mean to $\pm$ s.e.m. (N = 6, at all points.)

Thus in all three brain regions the pattern of labelling of acid-insoluble material follows the general pattern expected from a precursor-product relationship (ZILVERSMIT et al, 1943).

The results of the effects of exposure of birds to an imprinting stimulus or to diffuse overhead light on the ^{14}C-uracil incorporation into acid-insoluble and soluble material are shown in Figs 22 & 23. Because the average rate of incorporation varied from batch to batch, with consequent effects on the scatter of the data, the results had to be normalised. To correct for variations in bird weight, which would affect the absolute amount of ^{14}C-uracil present in the brain even with a constant amount injected, the specific activities of both the AI and AS fractions were corrected to what they should have been at a theoretical body weight of 50g. This was done by multiplying the specific activities for each bird by the factor $\left(\frac{50g}{\text{actual weight in g}}\right)$ derived from its real weight. (The normal range of bird weights was 40 - 55g.) To correct the data for batch variation, the AI and AS results from each batch were normalised about the mean of the AI and AS values respectively for all the brain regions of all the D birds in that batch, this mean being given the arbitrary value of 1.000.

This experiment on the incorporation of ^{14}C-uracil into RNA as a function of exposure of the chicks to stimuli was a

repeat of the earlier ^{3}H-uracil experiment by Bateson, Rose and Horn (1972). Consequently it was felt that to show the trends which had occurred in the incorporation of uracil into the different brain regions of the various groups of birds was sufficient, and that the pursuit of rigorous statistical significances in the results was unnecessary. Only twelve birds were used in each group (drawn from six hatches) and probability levels of less than 0.1 were taken as acceptable.

A slightly different series of changes in the incorporation of ^{14}C-uracil into RNA were found than had been reported earlier in the incorporation of ^{3}H-uracil (Figs 6 and 22 respectively). In the forebrain roof of the stimulus-exposed birds there was an elevation in ^{14}C-uracil incorporation after 38 minutes of treatment as compared to the dark-maintained birds ($\frac{38E}{D} = 1.14$; $P \leq 0.08$) but not to the diffuse light-exposed birds. This increased incorporation was maintained up to 76 minutes' exposure ($\frac{76E}{D} = 1.15$; $P \leq 0.08$), and a fall in incorporation in the diffuse light-exposed birds between 38 and 76 minutes ($\frac{38L}{76L} = 1.17$= $P \leq 0.09$) resulted in a large difference between the two exposed groups at the later time ($\frac{76E}{76L} = 1.33$; $P \leq 0.03$).

By contrast, in the ^{3}H-uracil experiment, although there was no incorporation difference between the dark-maintained and diffuse light-exposed birds in the forebrain roof at either time, there was also no decrease in incorporation in the latter group over the period 38 to 76 minutes. The stimulus-exposed groups also reacted somewhat differently in the two experiments.

Whereas in the ^{14}C- experiments there was an increase in incorporation in the forebrain roof of the E birds as compared to the D birds after 38 minutes, there was no difference between these groups at this time in the ^{3}H experiment and the increased incorporation in the E birds appeared only after 76 minutes. The magnitude of the changes was approximately the same in both experiments ($^{14}C \cdot \frac{76E}{D} = 1.15$; $^{3}H \cdot \frac{76E}{D} = 1.17$). If the earlier appearance of a difference in incorporation between the E and D birds in the ^{14}C experiment than in the ^{3}H one were the result of the earlier onset of the same train of biochemical events, then from the data on ^{3}H-uracil incorporation after 150 minutes of exposure, where in the forebrain roof $\frac{E}{D} = 1.33$ (BATESON et al, 1972) one would have expected that the difference between E and D birds at 76 minutes would be greater than at 38 minutes. This was not so.

The decline in incorporation of ^{14}C-uracil in the diffuse light birds was a whole forebrain phenomenon; in the forebrain base between 38 and 76 minutes of treatment the incorporation fell ($\frac{38L}{76L} = 1.18$; $P \leq 0.09$). Again this decline was not found in the forebrain base of diffuse light-exposed birds in the ^{3}H experiment.

In both experiments there were no differences in uracil incorporation into the midbrain between the five groups of birds.

Fig 23 shows the acid-soluble, i.e. free pool radioactive uracil, specific activity in the five groups of birds in the ^{14}C experiment. There were no differences in this parameter between any of the groups. However the variations between birds in the amount of free radioactivity in the brain was

quite considerable, as the data shows. This variation was not reflected in the incorporation data, suggesting that there were no rate-limiting effects due to uracil availability in the brain, although this does not rule out constraints due to the more immediate precursors of RNA, such as UTP. In the absence of information as to the specific radioactivity of the immediate RNA precursors, calculations of relative specific activity, i.e. (AI dpm/mg protein/AS dpm/mg protein), yield little in the way of useful data. This is especially true when, as here, the added precursor is several steps removed from the final precursor. Also the measurement of pool size at the end of the period of incorporation necessarily cannot take into account the fact that it may vary greatly within the incorporation period. It is quite likely that the pool size will have altered between the end of the exposure period and the time of killing (56 and 37 minutes respectively for the 38 and 76-minute treated birds).

The discrepancies between the two different isotope experiments in the incorporation of uracil into RNA in the forebrain roof of stimulus-exposed birds and in the whole forebrain of diffuse light-exposed birds remain unresolved. They may be due to a qualitatively different reaction to the diffuse light situation by the birds used in the two experiments. If so this would leave future studies open to serious doubts as to their reproduceability. However, it has been generally noticed that, within reasonable experimental variation, batches behave in a quantitatively similar manner, both with respect to behaviour and biochemistry. This suggests that there was some factor(s) in the diffuse light situation here which differed from that

of the prior Madingley experiment. Such variables are likely to be; temperature variations and differences, sound levels and the degree of social interaction between the chicks, which were not kept isolated from one another at all times, but in small groups. In subsequent experiments this was corrected by keeping the birds in individual pens at all times.

In spite of these discrepancies it was decided that the results of the two experiments were basically compatible, and that for complex behavioural experiments of this type general agreement was satisfactory. It would be undesirable, however, to conduct experiments which were mutually dependent without first ascertaining the results in both systems.

LEGENDS TO FIGURES 20-23.

Fig. 20 Specific radioactivity (dpm/mg protein) of acid-insoluble material in chick brain at several times after injection of ^{14}C-uracil. See Methods for details. Values are mean ± s.e.m. (N = 6).

Fig. 21 Specific radioactivity (dpm/mg protein) of acid-soluble material in chick brain at several times after injection of ^{14}C-uracil. See Methods for details. Values are mean ± s.e.m. (N = 6).

Fig. 22 Specific radioactivity (as % of mean of brain of dark-maintained bird) of acid-insoluble material after ^{14}C-uracil injection in three brain regions of chicks exposed to either an imprinting stimulus (solid lines) or diffuse light (dotted lines) or kept in darkness (on Y-axis). See Methods for details. Values are mean ± s.e.m. (N = 12).

a) forebrain roof	38E v D	$P \leq 0{\cdot}08$
	76E v D	$P \leq 0{\cdot}08$
	38L v 76L	$P \leq 0{\cdot}09$
	76E v 76L	$P \leq 0{\cdot}03$
b) forebrain base	38L v 76L	$P \leq 0{\cdot}09$
c) midbrain		

Fig. 23 Specific radioactivity (as % of mean of brain of dark-maintained bird) of acid-soluble material after ^{14}C-uracil injection in three brain regions of chicks exposed to either an imprinting stimulus (solid lines) or diffuse light (dotted lines) of kept in darkness (on Y-axis). See Methods for details. Values are mean ± s.e.m. (N = 12).

a) forebrain roof

b) forebrain base

c) midbrain

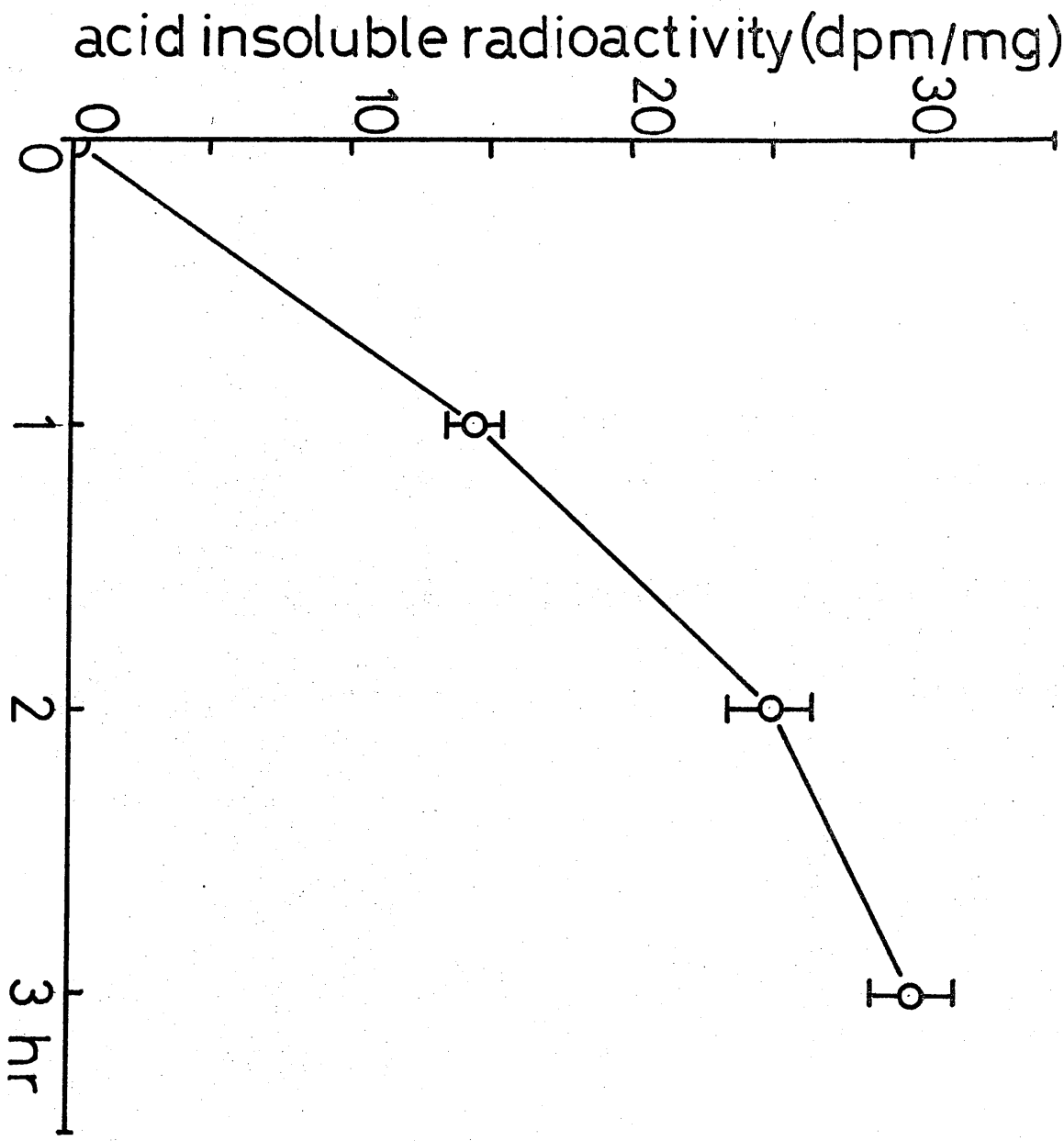


FIG. 20

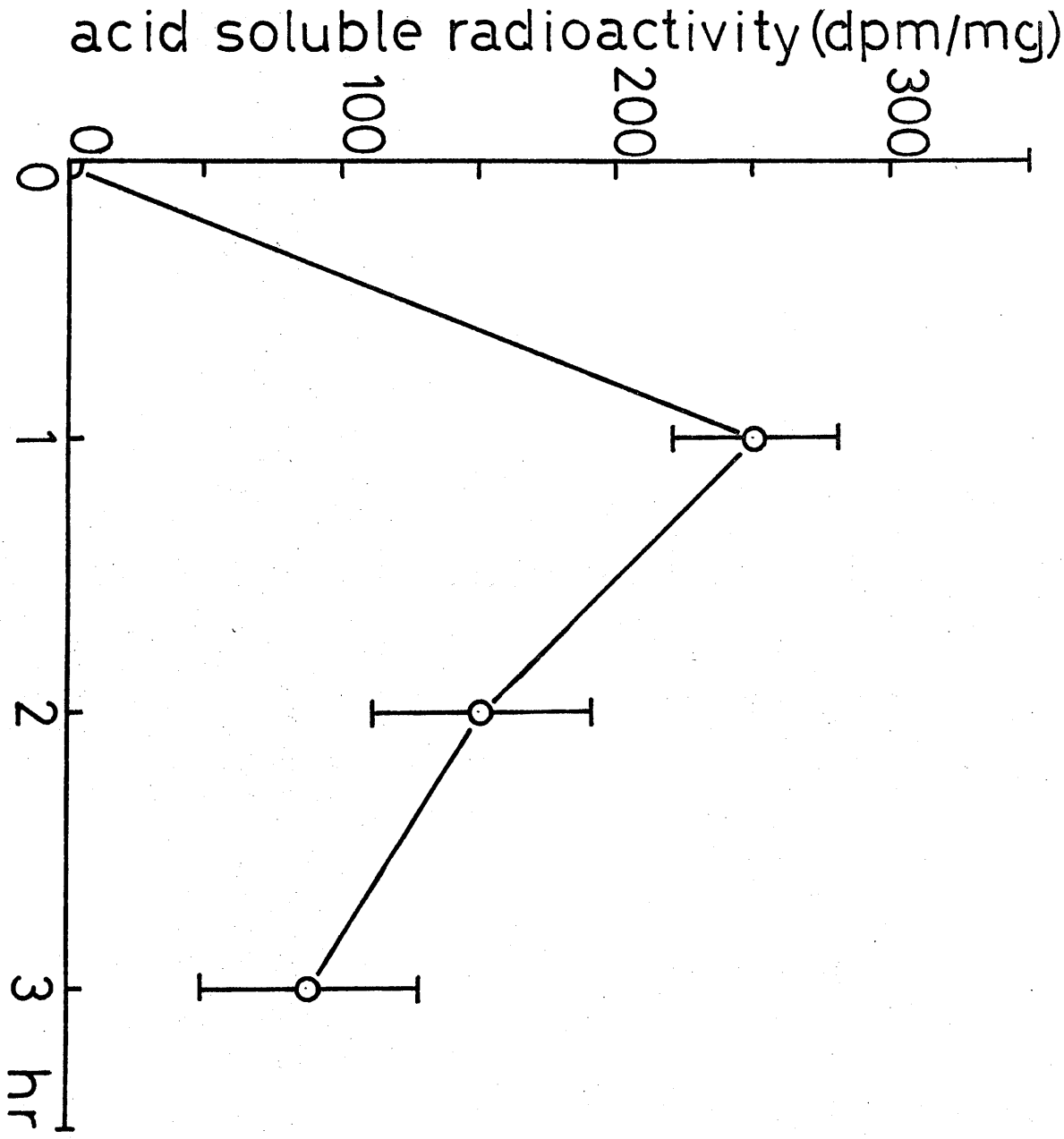


FIG. 21

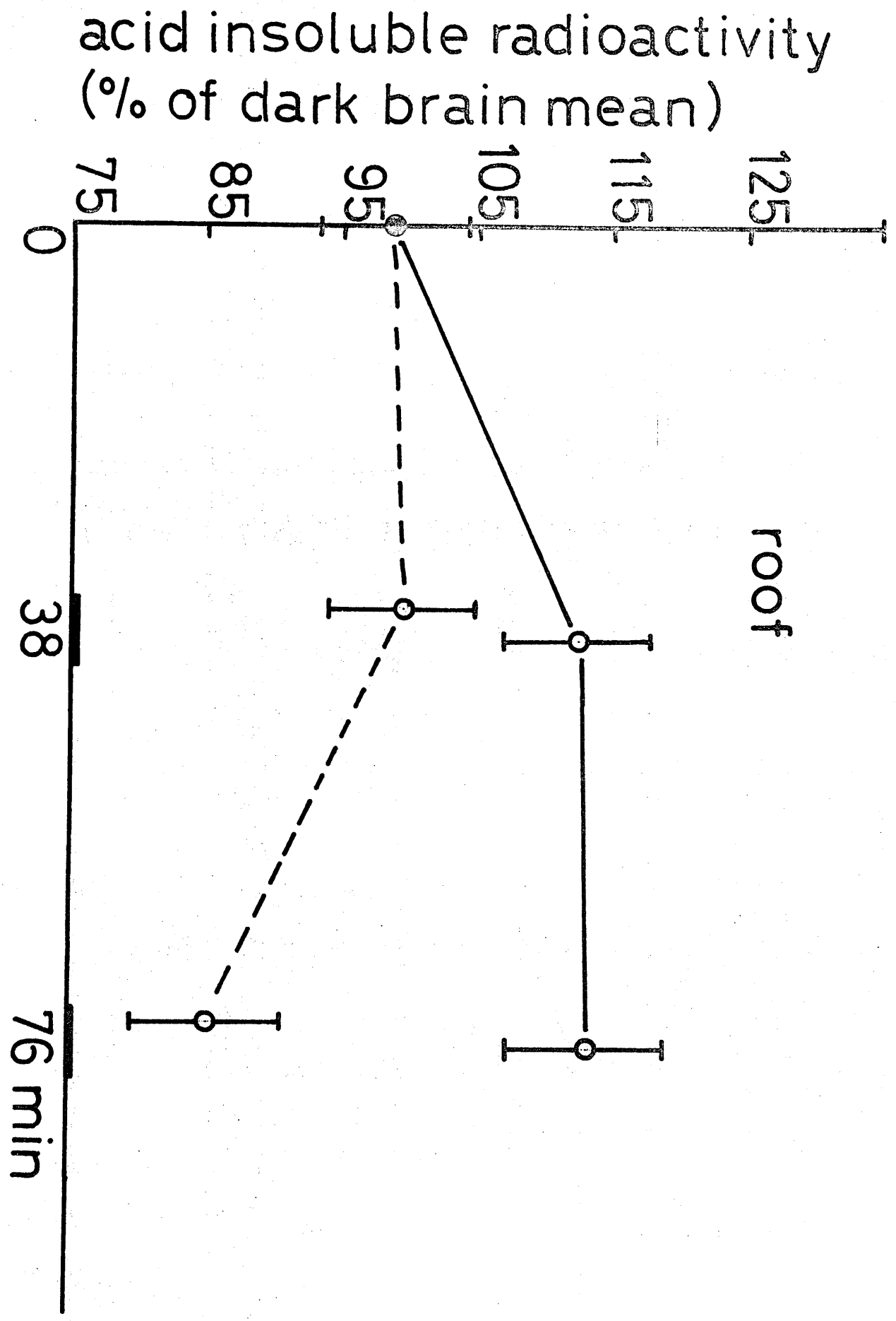

acid insoluble radioactivity
(% of dark brain mean)
75
85
95
105
115
125
0
38
76 min
roof

FIG. 22a

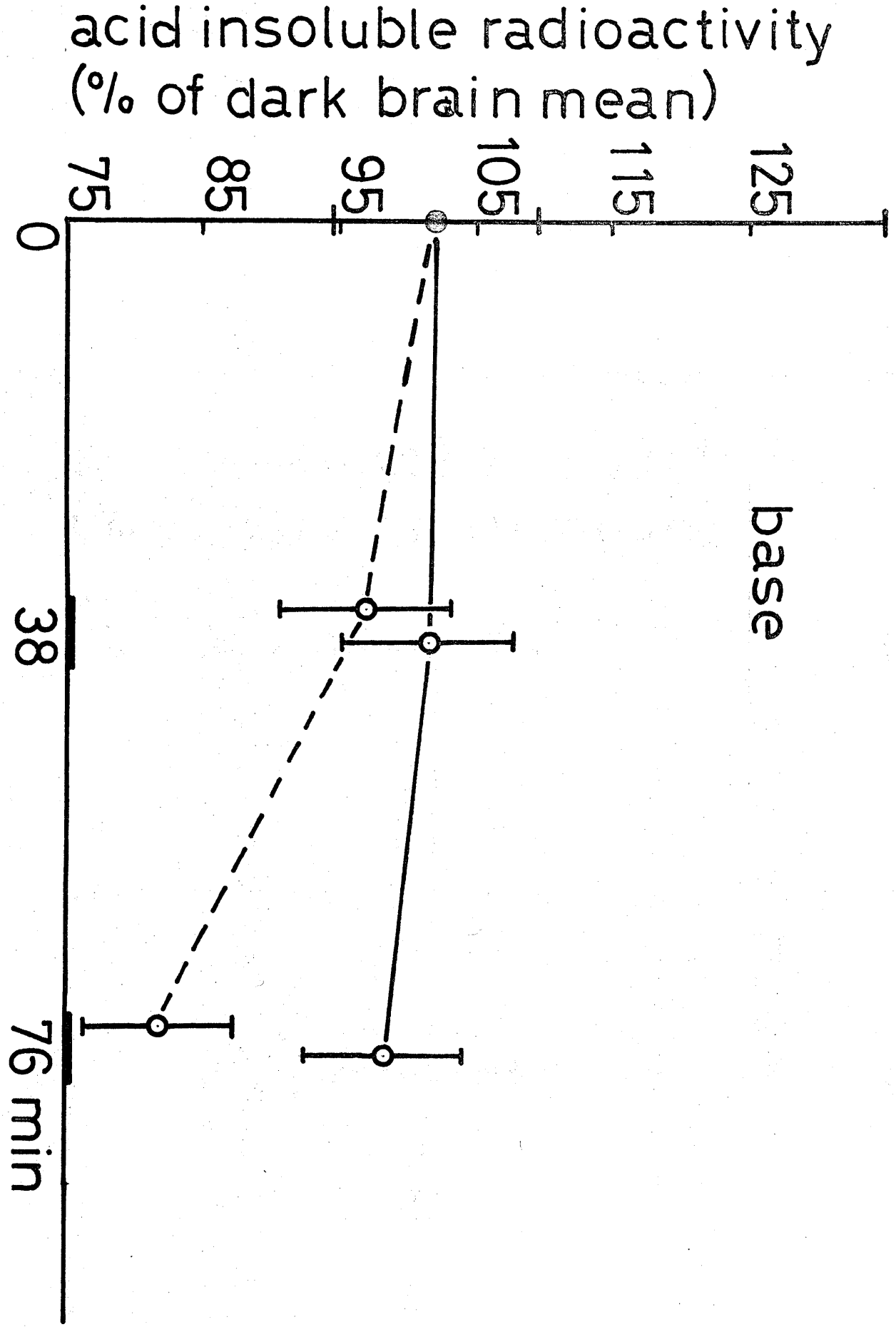

acid insoluble radioactivity
(% of dark brain mean)
75
85
95
105
115
125
0
38
76 min
base

FIG. 22b

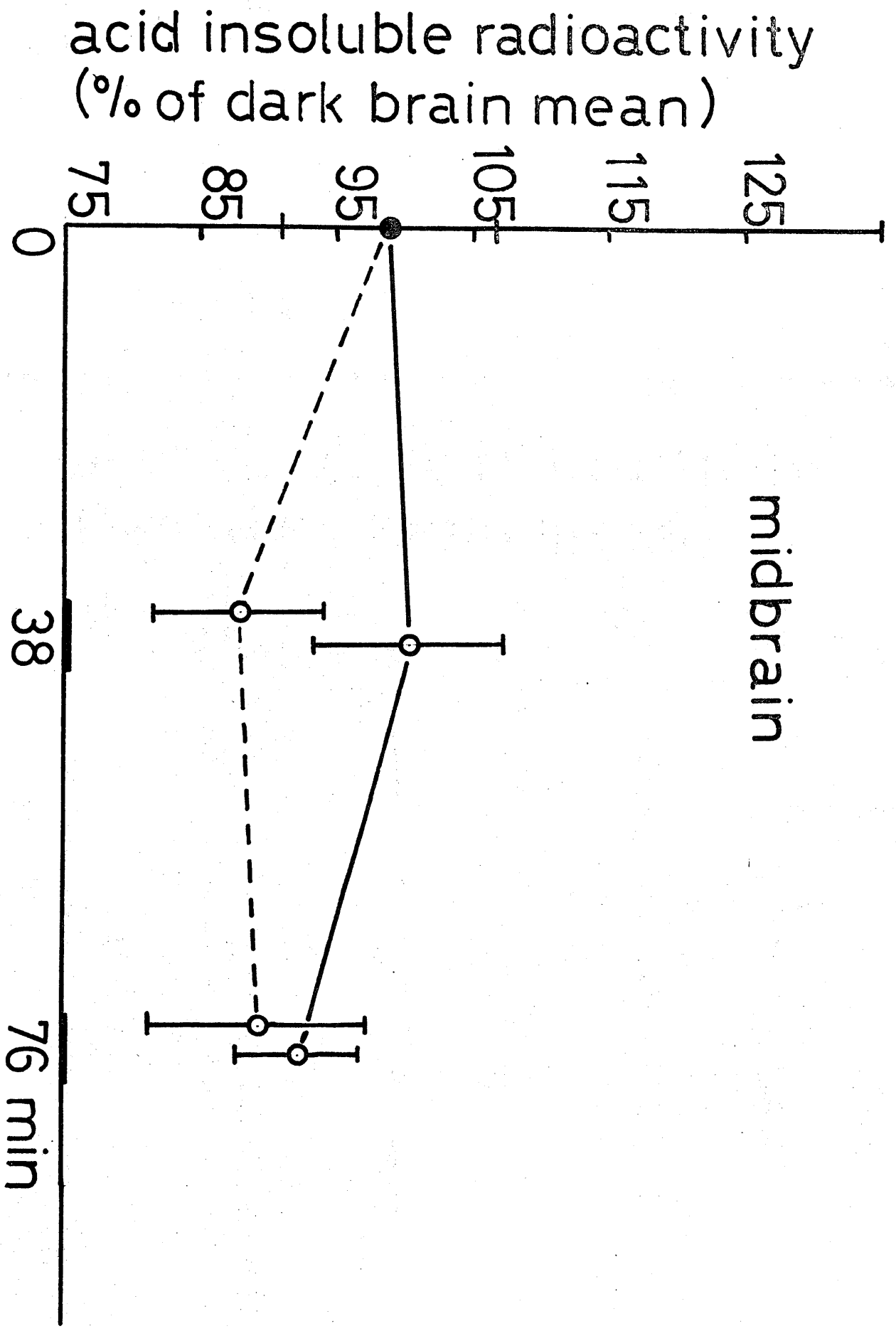

acid insoluble radioactivity
(% of dark brain mean)
75
85
95
105
115
125
0
38
76 min
midbrain

FIG. 22c

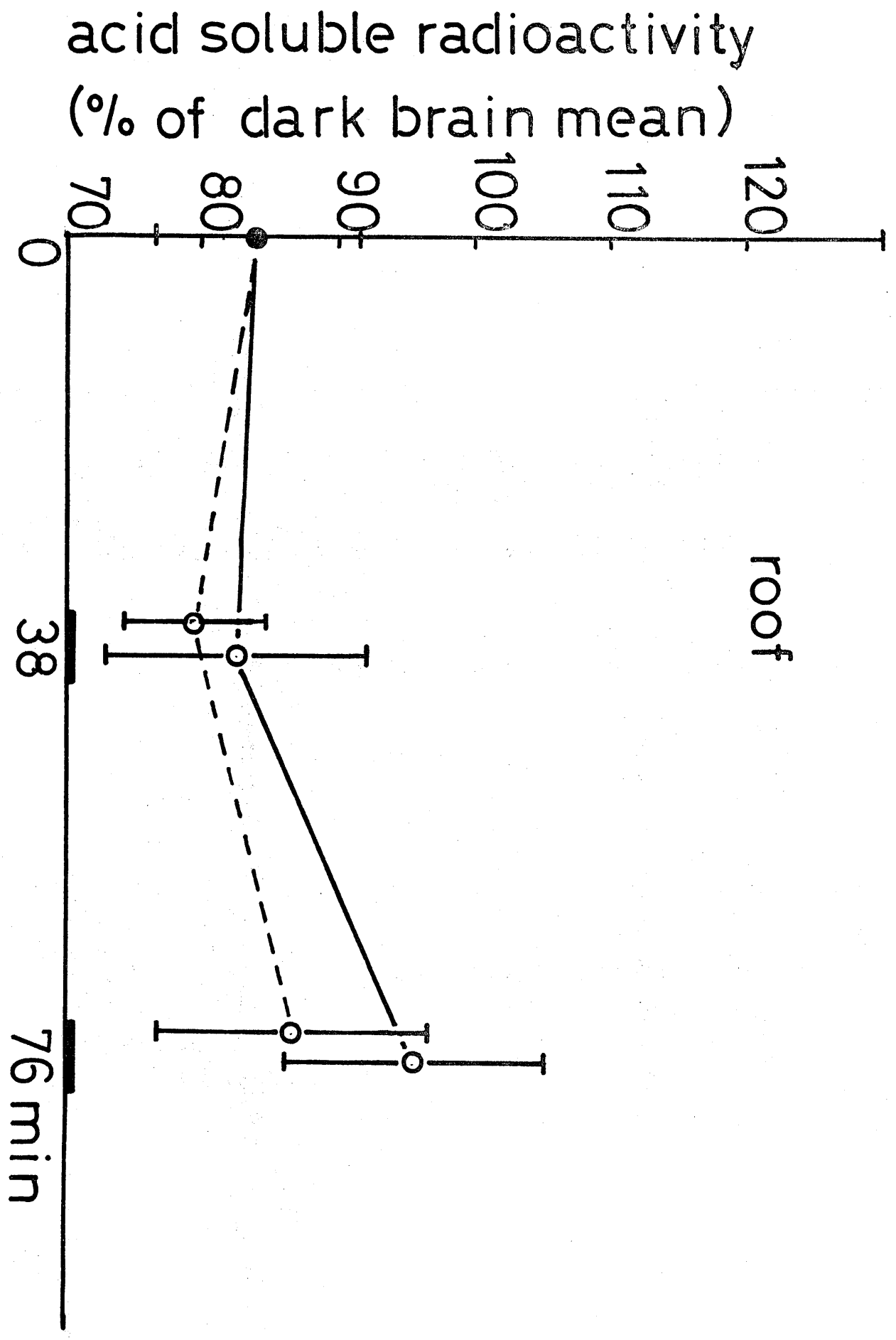

acid soluble radioactivity
(% of dark brain mean)
70
80
90
100
110
120
0
38
76 min
roof

FIG. 23a

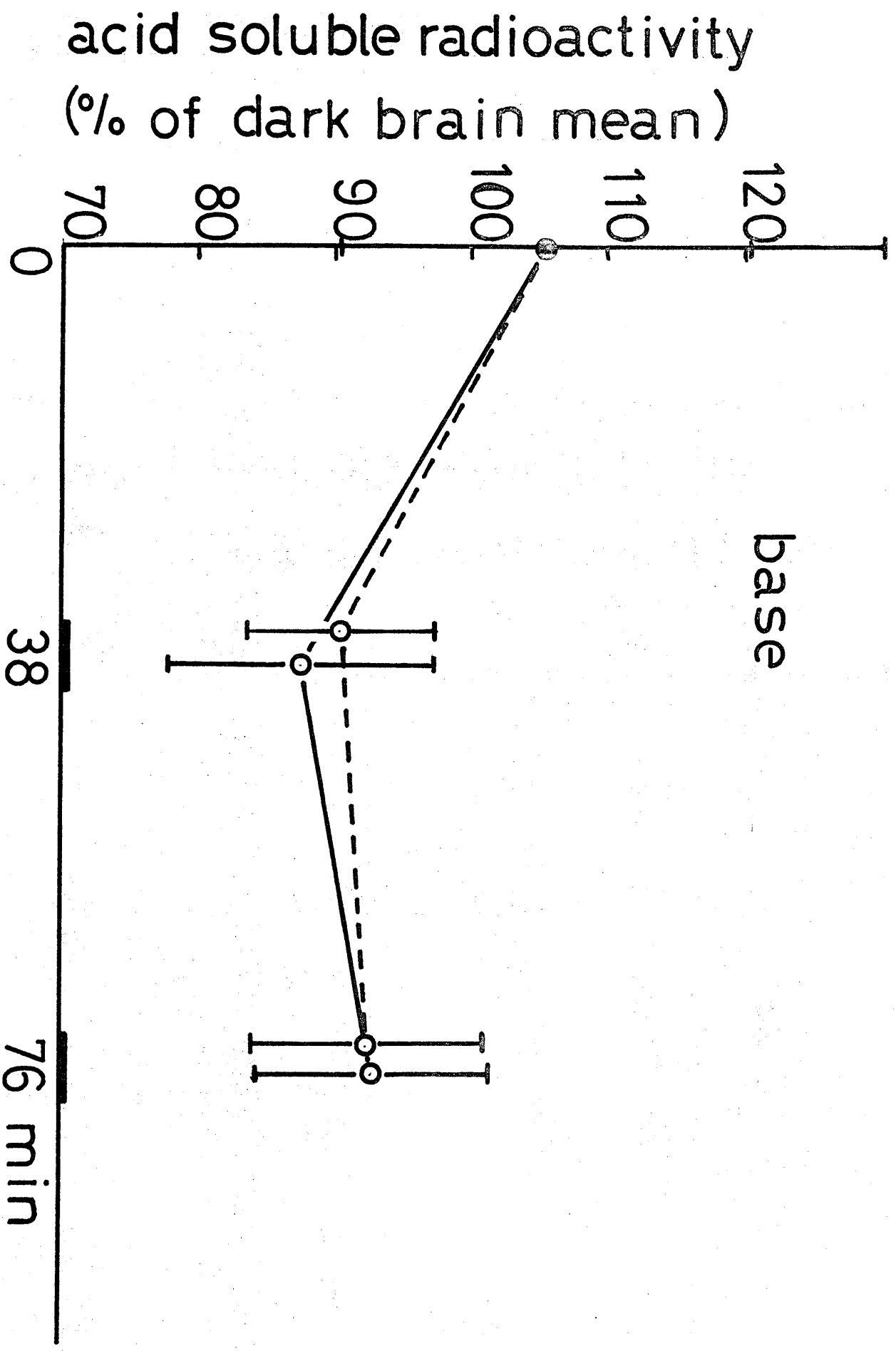

acid soluble radioactivity
(% of dark brain mean)
70
80
90
100
110
120
0
38
76 min
base

FIG. 23b

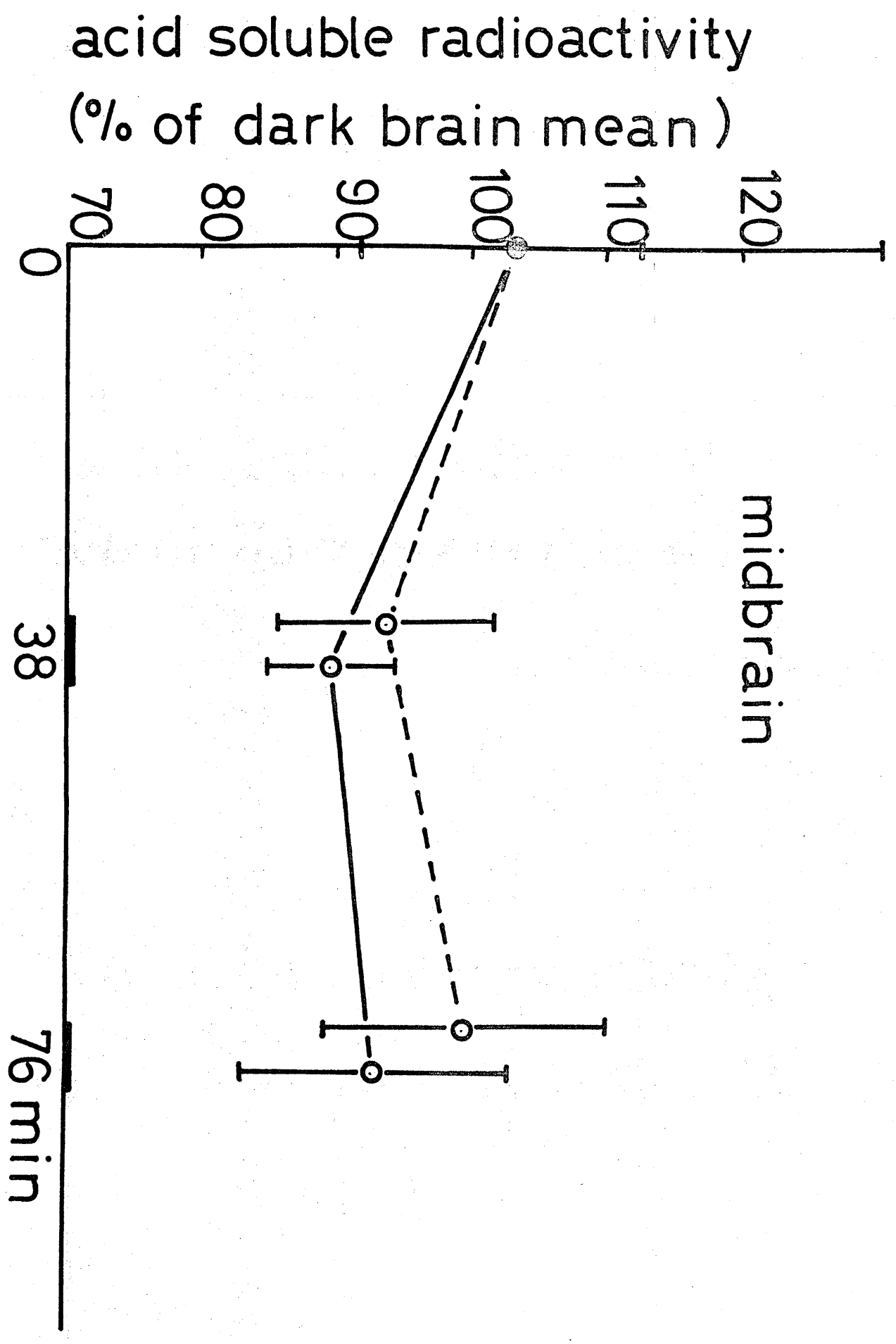

acid soluble radioactivity
(% of dark brain mean)
70
80
90
100
110
120
0
38
76 min
midbrain

FIG. 23c

CHAPTER VII

The changes in incorporation of ^{14}C-uracil into RNA in the one-day old chick brain as a result of varying periods of exposure to either diffuse light, an imprinting stimulus, or darkness had proven to be similar in some respects, but not all, to the incorporation of ^{3}H-uracil. In particular, the increased incorporation appeared at 38 minutes, rather than 76 minutes. This experiment had been the behavioural and biochemical 'test' of the imprinting equipment at Milton Keynes, and it was then appropriate to perform further experiments on the activity of RNA polymerase chosen as a consequence of the 30-minute preliminary experiment (Chapter V). After 30 minutes of exposure to the stimulus the activity of nuclear RNA polymerase in the forebrain roof of chicks was higher by 34% than that in unexposed birds, and in the forebrain base by 21%. It was therefore of interest to determine whether the same result could be obtained at Milton Keynes, i.e. confirmation of the earlier result, and to see whether any effects could be detected earlier than this time and for how long afterwards they lasted.

Materials and Methods:

Animals Ross I chicks were hatched and reared in isolation until 18 hours post-hatch as described before (Chapter V, p. 61).

Chemicals These were as used in the experiments in Chapters III and IV.

Behaviour Thirteen birds were taken from the early part of the hatch and divided one into each of thirteen groups:
naive, and a stimulus-exposed, a diffuse light-exposed and a

dark-maintained at each of four times: 15, 30, 45 and 60 minutes of treatment. The birds, except the naive which was left in the 33°C dark brooder incubator, were exposed to their respective treatments as shown in Fig 24. Because of the arrangement of the treatments the birds were all at the same mean age when killed, but differed slightly in their age at the onset of treatment. To reduce any order effects in the treatment of the birds, four 'schedules' were drawn up which were used to assign the order by which birds were introduced into, and removed from, the arena and dark-box. In a later series of experiments birds were treated for 120 minutes in each of the three conditions. The time required to test, kill and dissect a bird was four minutes and so the end-point of the whole experiment was not as sharp as indicated by Fig 24. The naive birds were not tested but were killed immediately on removal from the dark-brooder. The tissue samples were prepared, coded and frozen as described before (p. 64).

Preparation of nuclei and assay of RNA polymerase The nuclei were prepared and assayed for their RNA polymerase activity as described in Chapters III and IV.

Not all the samples (39) from each hatch could be worked up and assayed in one session and so those from one time point were worked up each day, the order in which they were done (i.e. 15, 30, 45 and 60) being randomised from week to week. The samples from the naive birds were worked up with one of the other time point samples.

Results:

Behavioural The median approach times for the birds in each category (except naive) are shown in Table 15.

TABLE 15

Median approach time
for each experimental type of bird

Bird Type	Duration of exposure to condition (minutes) 15	30	45	60
Stimulus-exposed (E)	64	53	31	>120
Diffuse light-exposed (L)	33	31	46	58
Dark-maintained (D)	>120	>120	>120	>120

Values are the medians of the times taken for each group of birds to approach to within 15cm of the stimulus-end of the alley (see Methods for details). The duration of the test for all birds was 120 seconds. In all cases except the 60E group the stimulus-exposed and diffuse light-exposed groups approached the stimulus faster than their corresponding dark-maintained groups. ($P \leq 0.02$ by Mann-Whitney U.)

None of the dark-maintained groups reached criterion, that is approached to within 15cm of the stimulus-end of the alley within the test time of 120 seconds. As had been observed before (BATESON et al, 1972), both the stimulus-exposed and diffuse light-exposed groups approached the stimulus more rapidly than the dark-maintained groups, although there was no significant difference between the two groups themselves. The exception to

this was the 60-minute stimulus-exposed group (60E) which contained several unresponsive birds and thus the median was raised to >120 seconds. Birds were not rejected for enzyme assay from any of the stimulus-exposed groups if they did not approach the stimulus within the allocated time because it was felt that their responsiveness in 2 minutes in the alley was not a sufficient indicator of their degree of imprinting. Moreover, even had the test been a more adequate measure of the extent of imprinting there is a very good reason for not rejecting birds on the basis of their behaviour in the test, namely that merely inactive birds in the E groups may be rejected whereas they would not be from the D groups which normally show little response. Thus the E population will be selected for high activity whereas the D population will be mixed. If there is a correlation between overall motor activity and the biochemical measure, then a significant difference between the two populations could be created merely as a result of such a rejection procedure. There was good similarity between the 30-minute groups' approach data in this experiment and that obtained at Madingley with the same groups (Table 11). This was further confirmation that the two systems were similar in the behavioural changes produced in the birds.

RNA Polymerase Activity As found previously there was variation between hatches in their overall level of RNA polymerase activity and normalisation was necessary before the data could be analysed statistically. There were four different dark-maintained groups, instead of the single group in the uracil experiment, and so instead of normalising around the mean of

of the activity of one dark group the data from each hatch was normalised around the mean of the activities in all the samples in that hatch. This procedure tends to minimise any effects due to an abnormal response in any single bird. The results of this procedure are shown in Fig 25 and Table 16.

There were no differences in enzyme specific activity in the midbrain between any of the groups (Fig 25c), and this accords well with the results of the ^{14}C-uracil incorporation experiment, no differences in incorporation having been detected in the midbrain.

The earliest differences appeared, after 15 minutes' treatment, in the base of the forebrain (Fig 25b) where the RNA polymerase activity was higher in the dark-maintained birds than in either the diffuse-light- or the stimulus-exposed birds ($\frac{D15}{L15} = 1.40$, $P \leq 0.05$ and $\frac{D15}{E15} = 1.38$, $P \leq 0.08$). This may have been a response to the initial handling at zero time when the birds were transferred from the dark incubator at $33^{\circ}C$ to their respective conditions at $30^{\circ}C$. After 30, 45 and 60 minutes there was no enzyme activity difference between the three groups and the activity in the dark-maintained birds fell over the period 15 to 45 minutes ($\frac{D15}{D45} = 1.55$, $P \leq 0.03$). This suggested that the initial response to handling, if that is what the difference at 15 minutes represented, occurred only in the dark-maintained birds. Perhaps the stress associated with such treatment was greater in the dark-maintained birds than in those exposed to light, because in the latter case the transfer was related to easily identifiable changes in the environment. The dark-maintained birds experienced a sudden drop in temperature and an increase in noise

levels from other birds (i.e. the light-exposed birds) but remained in essentially similar conditions to those they had been in until transfer. In some respects this is analogous to the situation of yoked control which experiences some facets of the altered environment but not those facets which permit it to make allowance for them by increased attentiveness to the pertinent stimuli. This, however, is a rather anthropocentric analysis of the situation.

The majority of the changes in RNA polymerase activity were found in the forebrain roof region (Fig 25a), very much in accordance with earlier results. There were no differences in activity between the groups after 15 minutes' treatment in this region, in contrast to the forebrain base, but between 15 and 30 minutes two opposed changes occurred which resulted in large differences appearing after 30 minutes' treatment. The activity in the diffuse light-exposed birds fell by about 30% ($\frac{L15}{L30} = 1.28$, $P \leq 0.03$) and that in the stimulus-exposed birds rose by the very large increment of 45% ($\frac{E30}{E15} = 1.45$, $P \leq 0.005$). The difference between the stimulus-exposed and dark-maintained birds therefore was similar to that obtained in the 30-minute RNA polymerase experiment discussed above (Chapter V). This result, like the uracil incorporation results was reproducible to some extent, although not in its entirety because the elevation in activity in the base of the E over D birds was lacking in this second experiment. Over the period 30 to 45 minutes, there was a decrease in activity in the stimulus-exposed birds ($\frac{E30}{E45} = 1.35$, $P \leq 0.03$) whilst the activity in the other two groups stayed constant, and consequently after 45 minutes' treatment there were no differences between the three groups. No further changes in activity occurred between 45 and 60 minutes.

To examine whether all the changes in RNA polymerase activity which resulted from the exposure of the chicks were finished, the experiment in which all three groups were treated for 120 minutes was performed. The data was internally normalised against the mean of all the activities in the hatch, and the results are presented in Fig 26. These birds differed from those in previous experiments in that they were not tested for approach responses at the end of their 120-minute treatment period, but as no differences in activity were found in the 45 and 60-minute birds it was felt unlikely that this would affect these results in any significant way. Although the percentage distributions of the enzyme activities are not appreciably different from those of the time course, they are not strictly comparable with them because the birds were drawn from entirely separate hatches.

There were no differences in enzyme activity between the three groups of birds treated for 120 minutes in any of the brain regions, and this was similar to the results obtained after 45 and 60 minutes. Thus from 45 to 120 minutes the activity of RNA polymerase remained constant in all regions of the chick brain irrespective of the conditions in which they were kept. All the changes which were found occurred within the first 45 minutes of treatment. It was fortunate that in the preliminary study a short period of exposure had been chosen.

DISCUSSION

It is not possible to make a simple, direct comparison between the data obtained from the ^{3}H- and ^{14}C-uracil incorporation experiments and those from the RNA polymerase activity experiments presented here. The uracil experimental design,

although it had varying periods of treatment for the diffuse light- and stimulus-exposed birds, had only one kind of dark-maintained bird (Fig. 5), namely one which had remained in darkness for the whole 150 minutes of the incorporation. Consequently these birds did not experience handling during the period of incorporation, at those times when the light-exposed groups of birds did. In this RNA polymerase experiment there is a matched group of dark-maintained birds for every treatment time, e.g. 45D for 45E and 45L, and these are not comparable with those of the uracil experiments. Indeed the response found in the forebrain base enzyme activity of the dark-maintained birds after 15 minutes suggests that they were very sensitive to such a manipulation as transfer. The uracil dark-maintained birds may have associated handling with injection: quite a stressful event.

It is more reasonable to make a comparison using the naive birds of the RNA polymerase experiments and the dark-maintained birds of the uracil experiments as being equivalent. Neither group was handled during the experiment for a transfer operation, and neither was exposed to the conditions of the imprinting room. The dark-maintained birds did have some differences in treatment from the naives, namely that they were kept for 150 minutes (the incorporation period) at 30°C and were handled and injected at the start of this period. This latter experience however was common to all groups of birds in the uracil experiments and might be expected not to create differences between them in respect of their uracil incorporation.

Alternatively one can compare only the data from the light-exposed groups, not taking the various dark groups into account. The major difficulty of interpretation which arises in doing so

is a result of the difference between the designs of the uracil and the RNA polymerase experiments. In both experiments there should have been no difference, behavioural or biochemical, between any of the birds prior to the onset of exposure, although of course the uracil birds had been handled and injected and could have differed as a group from the RNA polymerase birds which had no such experience. Aside from this, the birds received identical experiences whilst in the arena and in their subsequent removal from it. From this time on the experiences of the uracil and RNA polymerase groups diverge. The enzyme birds were tested and killed immediately, whereas the uracil birds were replaced in the dark brooder for a period and then tested and killed. It is quite possible that the RNA metabolism, including RNA polymerase activity, of these uracil birds was modified as a result of this process and hence the interrelationship of their final uracil incorporation levels were significantly different from what they had been at the end of the exposure period.

For the first type of comparison, that is taking the naive birds of the enzyme experiment and the dark-maintained birds of the two uracil (^{3}H and ^{14}C) experiments as being equivalent, the relevant changes in the three biochemical parameters are shown in Table 17 (over page).

The increased RNA polymerase activity in the forebrain roof of the stimulus-exposed birds after 30 minutes over that of the naives was reflected after 38 and 76 minutes' exposure in the ^{14}C-uracil birds and after 76 minutes' in the ^{3}H-uracil birds by enhanced incorporation in the stimulus-exposed as compared to the dark-maintained birds. It is of interest that although the preliminary RNA polymerase (30-minute exposure only) and the ^{3}H-

uracil experiments were performed on the Madingley equipment and the RNA polymerase time course and ^{14}C-uracil experiments on the Milton Keynes equipment, the same enhanced enzyme activity in the forebrain roof was found in both enzyme experiments whereas the increased ^{14}C-uracil incorporation in the same region appeared earlier than that of the ^{3}H-uracil. The use of the two differently labelled uracil precursors in these latter experiments may have contributed to this discrepancy.

TABLE 17

Some selected differences in cerebral nucleic acid metabolism between birds exposed to visual stimuli (I)

Brain Region	Parameter	Ratio of parameter in groups
Forebrain roof	RNA polymerase	$30E/_{N} = 1.20$
	^{14}C-uracil (AI)	$38E/_{D} = 1.14$
		$76E/_{D} = 1.15$
	^{3}H-uracil (AI)	$76E/_{D} = 1.17$
Forebrain base	^{14}C-uracil (AI)	$76L/_{D} = 0.80$

Data is extracted from Figs. 6, 22, 25; the experimental conditions and abbreviations are detailed in Chapters V and VII.

There was no change in RNA polymerase activity in the forebrain base of the diffuse light-exposed birds as compared to the naives which corresponded with the decreased ^{14}C-uracil incorporation in those diffuse light-exposed as compared to the dark-maintained birds, however this decrease may have been related to precursor supply rather than to enzyme activity (see p.79).

No changes in either RNA polymerase or ^{3}H- and ^{14}C-uracil incorporation were found in the midbrain, in less than 120 minutes' treatment.

A discrepancy which still remains is that between the 150-minute treated birds in the ^{3}H-uracil experiment and 120-minute treated RNA polymerase birds. Whereas there was enhanced ^{3}H-uracil incorporation in all three brain regions of the stimulus-exposed birds, and in the forebrain roof and base of diffuse light-exposed birds as compared to the dark-maintained birds there was no evidence of such phenomena in the enzyme data. (This however does assume that the three groups in the enzyme experiment would not have differed from naives at this time, because no naive birds were produced in this experiment.) As no 150-minute treated ^{14}C-uracil birds were used it is quite possible that their relative uracil incorporations would have been quite different to those found in the ^{3}H-uracil experiment; indeed massive trend reversals, particularly in the diffuse light-exposed birds in forebrain roof and base would have been needed for the ^{14}C-uracil data to have reached comparability with that of ^{3}H-uracil at this time.

For the second type of comparison, that is between the other two types of bird, the light-exposed groups, the midbrain is the simplest region in which to start. There were no differential changes in either ^{3}H- or ^{14}C-uracil incorporation

in either the 38- or 76-minute treated birds and none also in their RNA polymerase activity. This finding suggests that the two approaches in vivo and in vitro, to the effects of such treatments on nucleic acid metabolism in the brain do not differ qualitatively. To determine how quantitative their relationship is requires a knowledge of the magnitude of the RNA synthesis, which is not possible from these data.

The data relating to the following discussion is shown in Table 18.

TABLE 18

Some selected differences
in cerebral nucleic acid metabolism
between birds exposed to visual stimuli (II)

Brain Region	Parameter	Ratio of parameter in groups
Forebrain roof	RNA polymerase	$^{15L}/_{30L} = 1.28$
		$^{30E}/_{15E} = 1.44$
		$^{30E}/_{30L} = 1.44$
		$^{30E}/_{45E} = 1.37$
		$^{15L}/_{15E} = 1.17$
	^{14}C-uracil (AI)	$^{76E}/_{76L} = 1.33$
		$^{38L}/_{76L} = 1.17$
	^{3}H-uracil (AI)	$^{76E}/_{76L} = 1.17$

TABLE (cont.)

Brain Region	Parameter	Ratio of parameter in groups
Forebrain base	^{14}C-uracil (AI)	$^{76E}/_{76L} = 1.20$
		$^{38L}/_{76L} = 1.18$

Data is extracted from Figs. 6, 22, 25; the experimental conditions and abbreviations are detailed in Chapters V and VII.

There were no differences in RNA polymerase activity between the stimulus- and diffuse light-exposed birds in the forebrain base after any period of treatment. This is in agreement with the ^{3}H-uracil incorporation data, in which no changes were found at either 38 or 76 minutes' exposure. However, it does not agree with the ^{14}C-uracil data in which, between 38 and 76 minutes' treatment, the incorporation in the diffuse light-exposed birds fell. No changes which could have corresponded to this depression of incorporation were found in RNA polymerase activity over any time period. However there is no evidence to suggest that all the alterations in uracil incorporation must result from changes in RNA polymerase activity, other factors could result in such changes (see Chapters II and VI and later this chapter). In all three experiments the forebrain base, like the midbrain remained relatively unreactive, the major exceptions being the very early effects in the RNA polymerase activity in the dark-maintained birds and the decline in ^{14}C-uracil incorporation in the diffuse light-exposed birds.

This evidence of the general unreactivity of the forebrain base and midbrain contrasts sharply with the changes which occurred in both uracil incorporation and RNA polymerase activity in

the forebrain roof. In terms of uracil incorporation the stimulus-exposed birds had increased as compared to the diffuse light-exposed birds after 76 minutes' treatment. The changes in RNA polymerase activity occurred much earlier. After as little as 15 minutes' treatment the activity in the diffuse light-exposed birds was 30% higher than in the stimulus-exposed birds, but by 30 minutes this position had been reversed and the stimulus-exposed birds' activity was 45% higher than that of the diffuse light-exposed birds. By 45 minutes' treatment and from then until at least 120 minutes there were no differences between them, the activity in the stimulus-exposed birds having fallen to the same value as that of the diffuse light-exposed birds. Thus the nett RNA synthesis difference in the stimulus-exposed birds from 45 minutes onward would probably be higher than that in the diffuse light-exposed birds (and most certainly than that of the naives).

If these arguments are correct in attributing the increase in ^{14}C-uracil after 38 and 76 minutes and in ^{3}H-uracil after 76 minutes to increased RNA polymerase activity after 30 minutes' treatment, then the data cannot easily explain the decline in incorporation in the diffuse light-exposed birds over the period 38 to 76 minutes. The decrease in RNA polymerase activity in the diffuse light-exposed birds over the period 15 to 30 minutes was synchronous with the increase in activity in the stimulus-exposed birds. However, the appearance of the alterations in uracil incorporation were not synchronous. The decline in incorporation of the diffuse light-exposed birds occurred <u>later</u> than the increased incorporation in the stimulus-exposed birds. As noted earlier (p.79), there was a decline in ^{14}C-uracil incorporation

in the forebrain base between 38 and 76 minutes but no changes in RNA polymerase activity which could be attributed as causing this change. Because the two changes are of similar magnitude it seems reasonable to propose that there was a whole forebrain decline in uracil incorporation which was unrelated to the activity of RNA polymerase, but which was perhaps due to such factors as altered blood flow or cell permeability (see for example the work of BONDY, 1974, where blood flow changes occurred in response to visual stimulation). It is interesting that there was no such decline in ^{3}H-uracil incorporation in either of these brain regions in the earlier experiments, and so perhaps there was some factor present in the treatment of the diffuse light-exposed birds which was not constant between the two uracil experiments. This may have been during both or either of the exposure or darkness periods.

Possible significances of these results

These observations of changes in RNA polymerase activity which correlated to some extent with the results of the uracil experiments indicated that the incorporation changes seen did relate to altered RNA synthesis and were not entirely the result of variations in the radioactive uracil's specific activity or in uracil supply via the blood stream. This still leaves two aspects of the situation unexplored, namely the nature of the RNA which was formed by the RNA polymerase over the exposure period during which changes in activity were measured, and the mechanism(s) by which the altered activity of the enzyme was effected.

As we discussed earlier (Chapter IV) this assay probably measures a combination of the activities of multiple RNA polymerases, whose products are essentially mRNA and rRNA. This being so, the changes measured in total RNA polymerase activity could be due to the altered activity of one or several enzyme forms; however these cannot be distinguished with the present assay system.

There are several possible consequences of such changes in enzyme activity. If there was an increased net synthesis of mRNA, this could occur with or without the use of previously untranscribable DNA i.e. de-repression. If no new types of mRNA were synthesised, simply an increased quantity of the previous types, then, so long as the other components of the protein synthetic mechanism were not rate-limiting, there would have been an increased synthesis of those proteins which were already in production. In other words the rate-limiting factor in the protein synthetic mechanism was the quantity of mRNA available for translation. In the case of the production of new types of mRNA, and hence previously unsynthesised kinds of protein, mRNA did not need to have been rate-limiting for altered protein synthesis to have occurred. The new types of protein would have been translated along with the old.

If the only change in RNA synthesis was increased production of rRNA, and hence presumably ribosomes, then if ribosomes were previously rate-limting, increased production of those proteins coded in the existing types of mRNA would be formed. If ribosomes were not rate-limiting for protein synthesis then no change in quantity or type synthesised would

have occurred. The possible results of these changes are shown in Table 19, but in the absence of information as to rate-limiting factors in the protein synthetic processes of one-day old chick brain cells, and as to whether or not de-repression occurred, further speculation would be fruitless.

TABLE 19

Type of RNA increased	De-repression	Rate-limiting	Protein result
mRNA	NO	NO	NO CHANGE
	NO	YES	INCREASED SAME TYPES
	YES	NO	NEW TYPE; SAME QUANTITY AS PREVIOUSLY
	YES	YES	INCREASED QUANTITY WITH NEW TYPE
rRNA	NO	NO	NO CHANGE
	NO	YES	INCREASED SAME TYPES
	YES	NO	NEW TYPES; SAME QUANTITY
	YES	YES	NEW TYPES; INCREASED QUANTITY

The question of whether the altered activity of total RNA polymerase was due to changes in the activity of one or more forms of the enzyme could be answered by measuring the incorporation of labelled nucleosides due to each of the forms in nuclei from birds treated as they were in the experiment described above.

The inhibitor, α-amanitin, could be used to block nucleoplasmic RNA polymerase II (LINDELL et al, 1970) thus allowing measurement of the activity of RNA polymerase I in the nucleolus. Alternatively the isolated nucleoli could be assayed for enzyme activity after disruption of the nuclei (as for example in BANKS and JOHNSON, 1973) although difficulties in obtaining sufficient material for assay from one chick brain region could necessitate the pooling of samples.

The observed changes in RNA polymerase activity could have been produced by two mechanisms, either increased activity of existing enzyme molecules (activation) or by the synthesis of extra enzyme molecules. Ideally one could decide whether activation was the mechanism by 'mixing' experiments in which disrupted nuclei with enhanced activity were mixed with others with low activity. If some 'activating factor' were present (in excess) then the final activity would not be the mean of the two maximized activities but equal to the higher of the two. In practice it would not be possible to achieve two such populations of nuclei because the variations in activity between different groups was very high; not all preparations from exposed birds had higher enzyme activity than all those for dark-maintained birds. It would be easier, although still difficult, to look for increased de novo synthesis of enzyme as measured by enhanced incorporation of radioactive amino acid into RNA polymerase. However, given that there were changes in enzyme activity detectable after only 15 minutes of treatment, and that these changes were transient, it seems more likely that activation rather than increased synthesis of enzyme was the mechanism responsible. Just how such activation could have been achieved is unclear,

although as metal ions have dramatic effects on the enzyme's activity (see Chapter IV) these are possible candidates for such a role. From the point of view of cellular economy this mechanism would be preferable. When RNA polymerase activity increases after in vitro administration of hormones to cells, the agents directly responsible seem to be isolable cytoplasmic hormone-binding factors (DAVIES and GRIFFITHS, 1973). Also, because the assay in our experiments was not performed on the isolated enzyme but carried out in situ, changes in the transcribability of the chromatin cannot be ruled out (CHURCH and McCARTHY, 1970; BONDY and ROBERTS, 1969).

Both RNA levels and RNA polymerase activity have been shown to vary over a 24-hour period, that is they have circadian rhythms, in nuclei from cerebral cortex of rats on a 12-hour light/12-hour dark cycle. These changes are synchronised and are enhanced in the periods of increased motor activity (MERRITT and SULKOWSKI, 1970). Rats on a similar cycle were found to possess circadian rhythmicity of lysine incorporation but with the zenith approximately 12 later than that of the RNA levels noted above (RICHARDSON and ROSE, 1971). None of the experiments described or referred to here on changes in protein and nucleic acid metabolism have been of long enough duration to detect diurnal effects, however the possibility of such does highlight the problems involved in the interpretation of data from experiments such as these in which several factors are compounded. The relationship of the biochemical changes found to the imprinted behaviour must be continuously evaluated, taking into consideration the other changes in behaviour and metabolism which were occurring at the same time. The one-day old chick, reared in

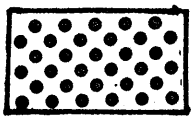
darkness, and then exposed to visual stimuli is in a phase of complex development, both physiological and behavioural. The light it receives is the first massive input to the visual system, and therefore probably results in either the onset or alteration of receptor and analytic processes, perhaps analogous to those found in the rat (ROSE, 1967; ROSE et al, 1973; ROSE and SINHA, 1974; CRAGG, 1969b). Visual stimuli with specific characteristics can produce specific behavioural changes ('imprinting') to which we have tried to relate the changes found in protein and nucleic acid metabolism, although even plain diffuse light, such as the diffuse light-exposed birds here received, modifies the birds' behaviour, particularly with respect to imprinting (BATESON and WAINWRIGHT, 1972). Synchronously with these effects there may be entrainment to a diurnal rhythm, which involves protein and nucleic acid metabolism. Of relevance here may be the possibility that these ubiquitous rhythms could be initiated and controlled by the transcription of DNA itself (EHRET and TRUCCO, 1967).

However, with these problems of interpretation taken into account, it does appear from the general internal consistency of all the data obtained on the biochemical responses of the chick after exposure to imprinting stimuli, that definite patterns of change in both protein and nucleic acid metabolism have been found which relate primarily to the acquisition of new behaviours.

(The results upon which this chapter is based have been published - Haywood et al, 1974c.)

LEGENDS TO FIGURES 24-26.

Fig. 24 Schematic representation of the operations performed to expose chicks for 15, 30, 45 or 60 minutes to either an imprinting stimulus, diffuse light or a dark-box. The temperature for all exposures was 30°C; for testing it was 26°C.


stimulus


diffuse light


dark-box

Fig. 25 Relative RNA polymerase activities in three brain regions of chicks exposed for 15, 30, 45 or 60 minutes to either an imprinting stimulus, diffuse light or a dark-box. Data is expressed as % of the mean enzyme activity of all the samples in each experiment. See Results for details. Values are mean ± s.e.m. (N = 12). All statistical analyses are shown in Table 16. Shadings are as for Fig. 24.

a) forebrain roof

b) forebrain base

c) midbrain

Fig. 26 Relative RNA polymerase activities in three brain regions of chicks exposed for 120 minutes to either an imprinting stimulus, diffuse light or a dark-box. Data is expressed as % of the mean enzyme activity of all the samples in each experiment. See Results for details. Values are mean ± s.e.m. (N = 12). Shadings are as for Fig. 24.

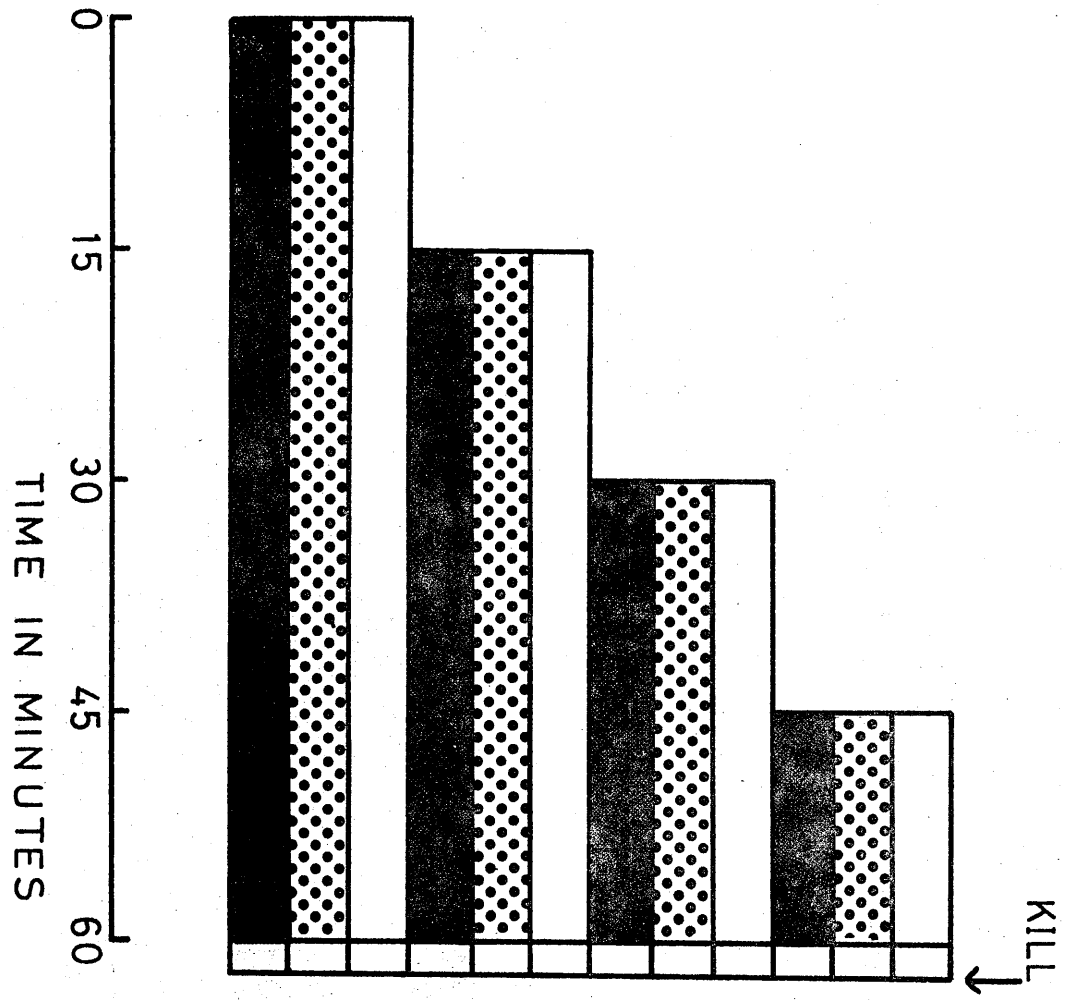


FIG. 24

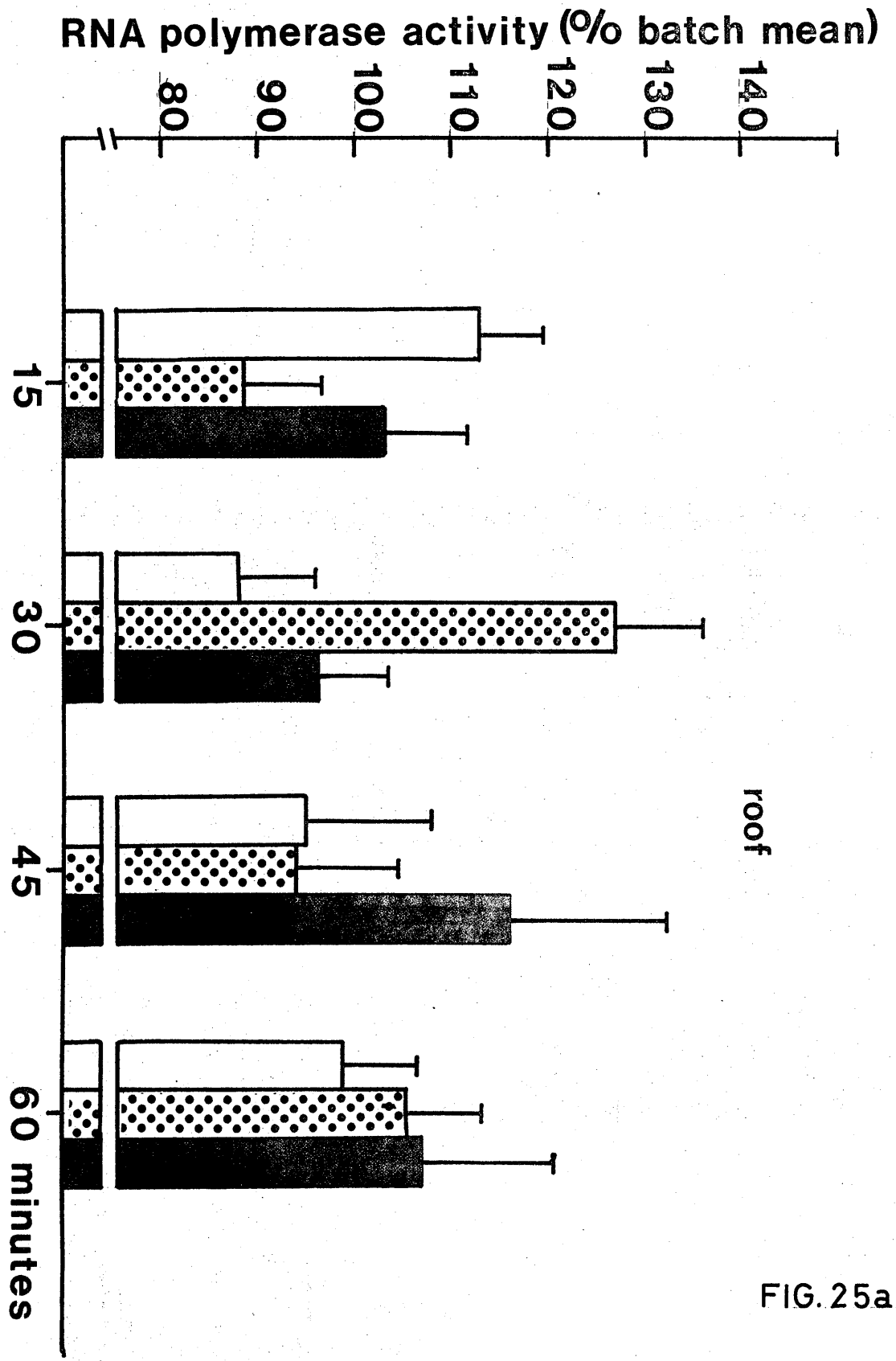

RNA polymerase activity (% batch mean)
80
90
100
110
120
130
140
15
30
45
60 minutes
roof

FIG. 25a

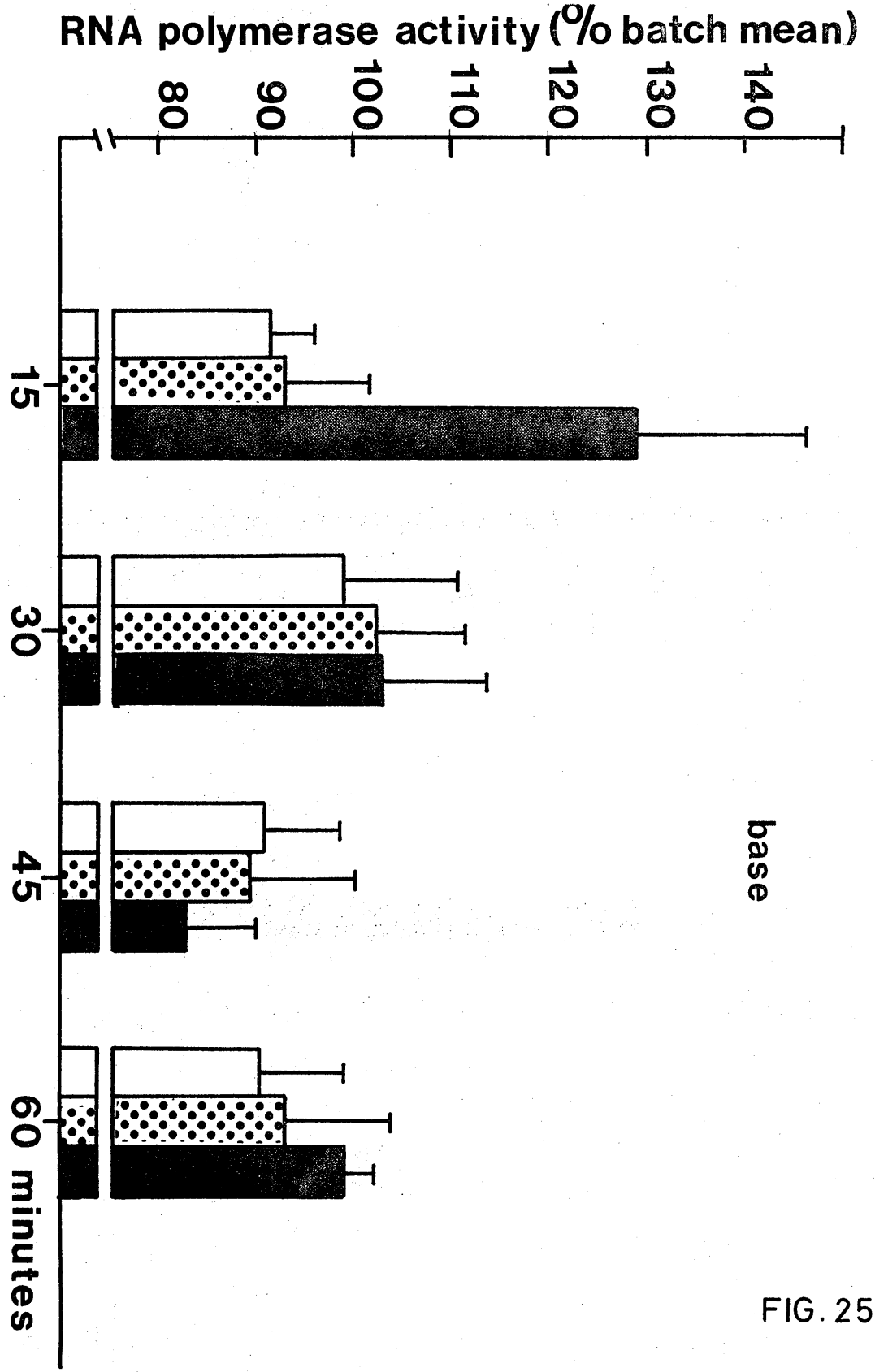

RNA polymerase activity (% batch mean)
80
90
100
110
120
130
140
15
30
45
60 minutes
base

FIG. 25b

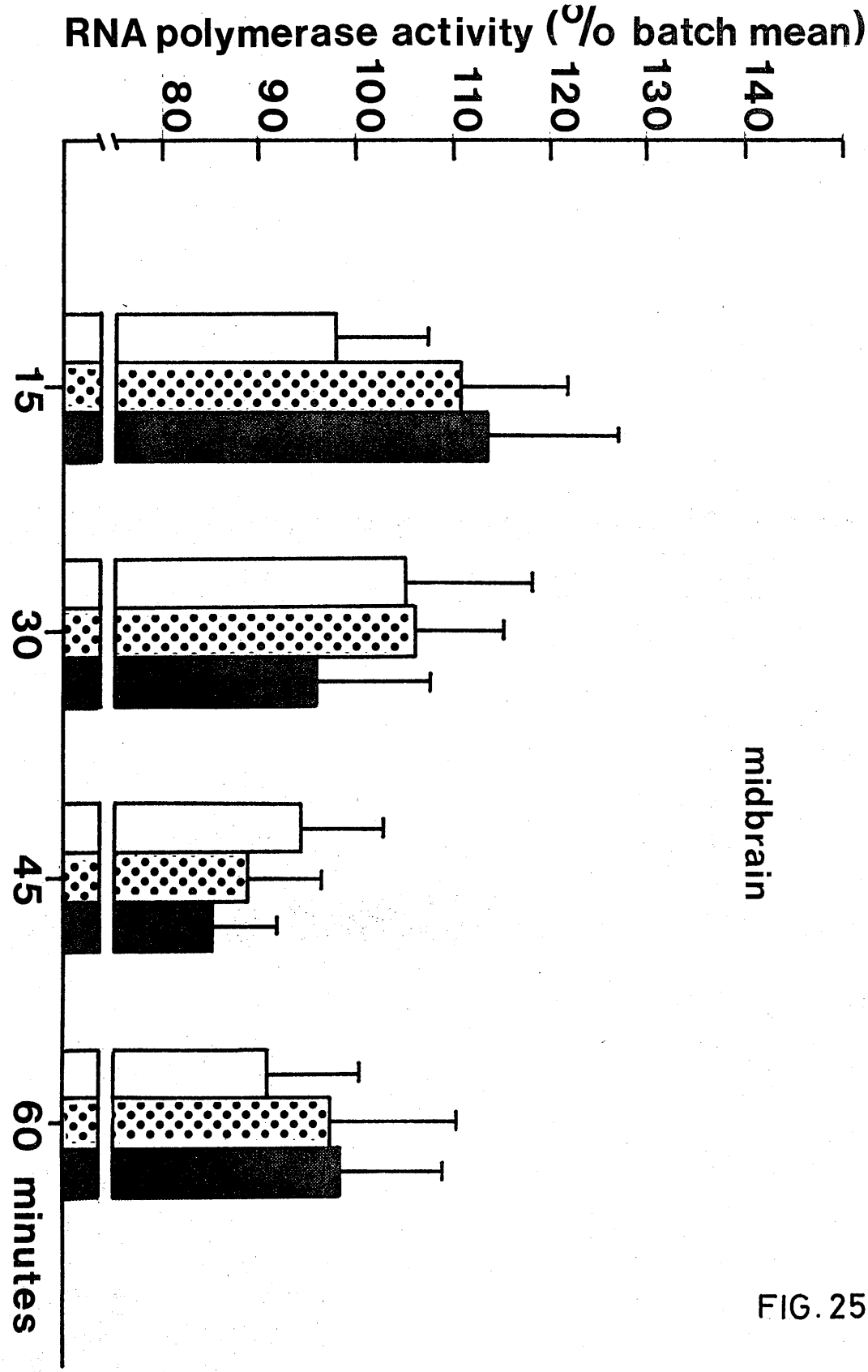

RNA polymerase activity (% batch mean)
80
90
100
110
120
130
140
15
30
45
60 minutes
midbrain

FIG. 25c

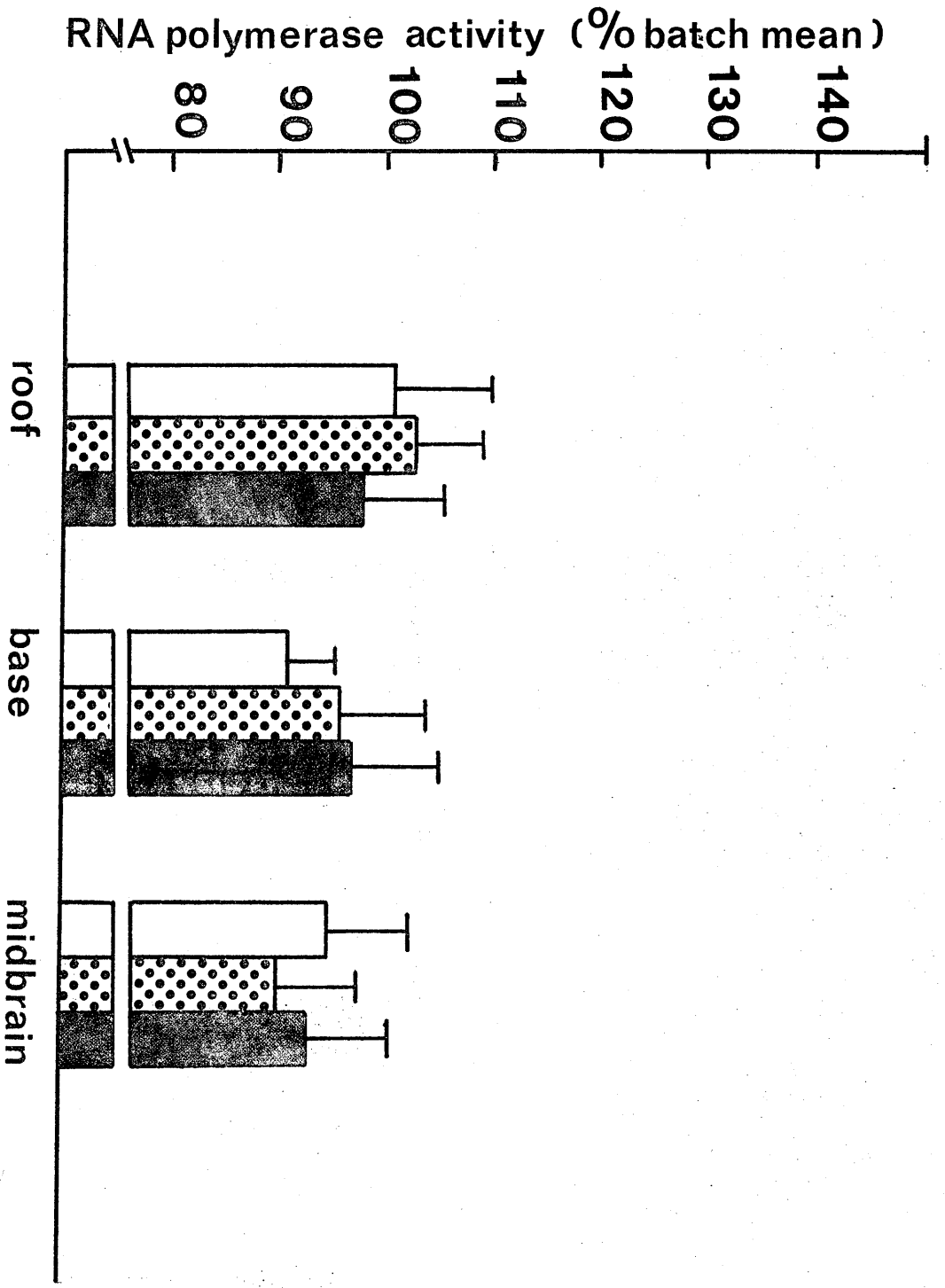

RNA polymerase activity (% batch mean)
80
90
100
110
120
130
140
roof
base
midbrain

FIG. 26

CHAPTER VIII

In Chapters I and II some of the current ideas which direct research into the neurochemical aspects of effects on stimuli on the brain, and in particular of macromolecular metabolism in relation to information storage, were discussed. The experiments which have been described here were designed to examine one aspect of such processes, namely the changes in nucleic acid metabolism in birds exposed to a situation in which they acquire specific behaviours. The activity of total RNA polymerase, that is, without discrimination between either its forms or its cellular distribution, was shown to change transiently following short periods of exposure to the imprinting stimulus. Also these changes in activity bore both a temporal and a qualitative relationship to the altered incorporation of uracil into RNA observed after longer periods of exposure.

There are several reasonable directions in which further research into these phenomena could go, not all of which directly involve nucleic acid metabolism.

Firstly there is an analysis of whether the altered enzyme activities represent differential changes in the activities of several enzyme forms (as mentioned in Chapter VII). If the enzyme forms in chick brain nuclei are essentially the same as those found in other systems, that is one produces ribosomal, and the other messenger RNA, then this would be an indication of increased production of one or both of these types of RNA. In the case where only increased production of

ribosomal RNA occurs then one can speculate that there is no alteration of the proportions of the types of protein being formed. However, no studies of this type can allow for translational control of protein synthesis, that is differential use of various messenger RNAs and so give only suggestive information about the nature of the end-products of the process, the proteins. Moreover, if the changes in enzyme activity are due to activation by small molecules, disruption of the nuclei, and subsequent purification of the nucleoli, could result in a loss of these activators and thus cast severe doubts upon the validity of the results. This is especially true where, as here, the differences in total activity were quite small.

Secondly, one could examine the products of the increased enzyme activity for increases in messenger and ribosomal RNA, probably measured as increased in vivo incorporation of labelled precursors rather than as absolute amounts. Some work in this direction has been reported by Coleman et al (COLEMAN et al, 1971). It is very unlikely that any differences within the messenger RNA population itself, that is indications of new species, could be detected because of the problems involved in DNA-RNA hybridisation studies (e.g. see CHURCH and McCARTHY, 1970; von HUNGEN, 1970 but also MACHLUS and GRITO, 1968, 1969).

Thirdly, the cell types from which the nuclei were prepared was not examined. Thus the enzyme activity which was measured related to a mixture of neuronal and glial nuclei. If differential total enzyme activities were present within these two major types of nuclei, or among the many different kinds of each, then knowledge of which types of cell nuclei

were responding under the exposure conditions would be extremely useful. The project is beset by difficulties, however. Without detailed information of the characteristics of the nuclei from all the cell types involved i.e. neuronal, astroglial, oligodendroglial, it is impossible to define the products of a nuclear fractionation procedure. Even in the rat, where more details are available, the identification is still ambiguous (see Chapter III). Also the quantity of starting tissue required for most separations, and in particular those in which RNA polymerase activity on the fractionated nuclei is to be assayed, is quite large (e.g. several forebrain roofs), the tissue from several experimental animals would be needed. Because of the number of animals available for exposure to the stimulus and the problems in preparing many nuclear samples from them, the process would be very difficult.

Nucleic acid metabolism, although of great interest for describing part of the mechanisms whereby brain cells respond to inputs, is unlikely of itself to yield at present much information about the changes in cell structure and function which underlie plasticity and thus perhaps information storage. Few workers believe that nucleic acids themselves are the agents which store information, rather that they are involved in such by virtue of their actions in the protein synthetic mechanism. Thus for investigations whose aim is to elucidate the changes in cell structure, that is to a great extent the structure and function of the synapse, analysis of the proteins formed as end-products in the reaction is likely to be more fruitful.

Nucleic acids cannot be analysed in terms of which proteins they code for, and even if they could, the possible intervention of translational control would prevent any description of the ultimate protein products by such means. It is therefore preferable to look at the proteins themselves, either by precursor incorporation methods or by assay of the activities of known enzymes. to see whether these are altered in response to stimuli.

The proteins of neurons appear to be synthesised either in the perikaryon (the major site) or in the synapse, and some of those which are of perikaryal origin are transported to the synaptic region. Thus by examining protein metabolism in isolated cell types and in synaptosomal preparations it is possible to define more closely the types of proteins in production and their final localisation (CUÉNOD et al, 1972). A further aspect of the metabolism of proteins which is also open to study by these methods is the modification of glycoproteins. Because some glycoproteins seem to be completed after the polypeptide chain is produced, and some even after the protein has reached the synaptic region (see for example BARONDES, 1969), there is the possibility of responses which do not involve changes in de novo protein synthesis but which do affect the proteins themselves. Also within this category is protein phosphorylation which probably involves cyclic nucleotides (MACHLUS et al, 1974) and which could be either nuclear, where it might affect RNA synthesis on DNA, or at the synapse and affect transmission.

Many investigations are now following these lines (ROSE and SINHA, 1974; MACHLUS et al, 1974; REES et al, 1974).

We have begun to examine some of these problems in relation to the biochemical responses of the chick brain to imprinting stimuli. (These experiments were performed in collaboration with: J. HAMBLEY, G. R. DUTTON, S.P.R. ROSE and P.P.G. BATESON). In chicks exposed to the stimulus for periods up to 2 hours we have detected changes in both protein and glycoprotein metabolism and in the activity of some enzymes involved with neurotransmitter metabolism. The only difference found in ^{14}C-lysine incorporation between exposed and dark birds was after 60 minutes' treatment, there being no difference after 30 or 120 minutes, and this effect was confined to the anterior part of the forebrain roof. We have subsequently found that this increase is mostly confined to the 6×10^6 g-min supernatant, the soluble fraction. For studying glycoprotein metabolism we injected ^{14}C-fucose either at the start of a 60-minute exposure or at the end of it, with a pulse time of 60 minutes. No difference was found between the stimulus-exposed and dark-maintained birds in fucose incorporation over the 60-minute period (in darkness) after the treatment, but there was increased incorporation in the stimulus-exposed birds during the incorporation, but only in the forebrain roof. In both the fucose and lysine experiments there was increased acid-soluble radioactivity in all brain regions of the stimulus-exposed birds but only the roof region showed increased incorporation. In the case of lysine, this increased free radioactivity did not produce a change in the specific radioactivity of the precursor pool.

Precursor incorporation studies do not give any indication of changes in specific metabolic processes related to brain function such as neurotransmission, and so we have also studied the responses of two enzymes involved with acetylcholine metabolism, acetylcholinesterase (AChE) and choline acetyltransferase (ChAc). We thought that the appearance of any such changes might be delayed with respect to the stimulation, particularly if protein synthesis and transport were involved, so we exposed birds for 60 minutes to the stimulus or kept them in the dark-box and then killed them after 0, 1, 6, 12 or 24 hours in a dark brooder. ChAc activity was increased in the midbrain of the stimulus-exposed birds immediately after the exposure period, but this had disappeared one hour later and no further differences were found. AChE activity did not change until 1 hour after the end of exposure, at which time it was increased only in the forebrain roof of the stimulus-exposed birds. This was followed by a general brain elevation in activity in these birds by 6 hours post-exposure. After 12 hours the only effect was a depressed activity in the midbrain of exposed birds and by 24 hours no differences were found. Thus a series of increases and decreases in the activity of AChE and ChAc occurred, several of which had long delays as compared to the nucleic acid protein and glycoprotein metabolism changes. Such data is suggestive of alterations in the perikaryal synthesis of proteins whose eventual site of action is synaptic, the delays being incurred during transport along the neuronal processes. (Some of these results have been or are being published: HAYWOOD et al, 1973; HAYWOOD et al, 1974a, b; HAMBLEY et al, 1974.)

The other end of the problem is the relationship between the nerve impulse and the observed changes in nucleic acid and protein metabolism. This can be studied at either the single synapse preparation level or in the whole animal using different stimuli to vary the input to the nervous system. Use of the former has the drawback that if only small changes of metabolism occur they will be difficult to detect; the latter that different cell populations may respond in different ways. Another difficulty associated with whole animal studies of very early neuronal events is associated with the length of exposure to the stimulus. In many training experiments the behaviour of the animals after very short treatments has not developed such that learning can be said to have occurred. For example in experiments where chicks are exposed to an imprinting stimulus their behaviour at short exposure times (up to about 10-15 minutes after onset) is more akin to stress response, and often has little element of orientation towards the stimulus. Thus there is no clear indication at which point the birds began to learn the characteristics of the stimulus. It may be that such behaviour is a necessary pre-condition for subsequent learning in such situations. There may be complex events occurring within the previously unused visual system which are also necessary pre-conditions for learning. Certainly, as mentioned before, previous exposure to plain light for short periods before exposure to the stimulus enhances their subsequent behaviour towards it (BATESON and WAINWRIGHT, 1972). Situations which involve one trial learning may thus be more appropriate for such early-event studies. (e.g. CHERKIN, 1969; WATTS and MARK, 1970).

If the response observed in these experiments of enhanced RNA polymerase activity, followed by altered incorporation of uracil into RNA and lysine into protein do represent changes of macromolecular synthesis in response to significant inputs to the CNS and are involved in the storage of that information, then at least one, and possibly several, processes may link these changes to the nerve impulse. The nerve impulse is a cell membrane effect, a selective uptake and a selective out-put of ions; RNA polymerase is localised within the nucleus, and associated with the chromatin. Thus somehow there must be an information flow between either the cell membrane, or the cytoplasm, and the nucleus, which affects at least RNA polymerase, and possibly the chromatin too. Phosphorylation of nuclear proteins has been shown to occur after very short training periods in mice, or as a result of 'reminding' (MACHLUS et al, 1974). Much attention has been paid recently to the role of cyclic AMP in the nervous system, some of which would suggest it as a possible candidate for such a role (e.g. LANGAN, 1969; SIGGINS et al, 1969). However in the best studied system where specific enzyme induction appears to occur as a result of stimulation, cAMP seems to have rather broader actions (THOENEN, 1974). It may instead be involved in modification of synaptic proteins and thus directly influence transmission (JOHNSON et al, 1972). Whether one or both of these effects turns out to be directly related to neuronal modification in response to stimuli, the speed with which the synthesising enzyme, adenyl cyclase, can respond makes it a good candidate for involvement in early events. (This is not to neglect its possible longer-term actions - see for example HAMBLEY and ROSE, 1974, where the largest increases in adenyl cyclase activity appear after long periods of exposure of chicks to an imprinting stimulus.)

In the first instance such links between the metabolisms of cyclic nucleotides and nucleic acid may most profitably be studied in isolated cell populations (i.e. neurones and glia) where the complications of different effects in different cell types can be reduced. If and when connections have been detected then the simple behavioural systems (such as one trial learning as mentioned above) could be used to test whether there were detectable differences in them in response to stimuli.

Both of the types of investigations outlined above, that is into short-term and long-term events, are possible directions in which studies of the effects of environmental stimuli on the nervous system could be reasonably pursued.

ADAIR, L.B., Wilson, J.E., and Glassman, E. (1968a) Proc. natn. Acad. Sci. U.S.A. 61:917-922. "Uridine incorporation into polysomes of mouse brain during different behavioural experiences."

ADAIR, L.B., Wilson, J.E., Zemp, J. W., and Glassman, E. (1968b) Proc. natn. Acad. Sci. U.S.A. 61:606-613. "Brain function and macromolecules III. Uridine incorporation into polysomes of mouse brain during short-term avoidance conditioning."

AGRANOFF, B.W. (1967) Rockefeller University Press, N.Y. "Agents that block memory." In: The Neurosciences: A Study Program. Eds: Quarton, G.C., Melnechuk, T., and Schmitt, F.O.

AGRANOFF, B.W., David, R.E., and Brink, J.J. (1965) Proc. natn. Acad. Sci. U.S.A. 54:788-793. "Memory fixation in the goldfish."

ANDREW, R.J. (1964) Anim. Behav. 12:542-548. "The development of adult responses from responses given during imprinting in the domestic chick."

AUSTOKER, J., Cox, D., and Mathias, A.P. (1972) Biochem. J., 129:1139-1155. "Fractionation of nuclei from brain by zonal centrifugation and a study of RNA polymerase activity in various classes of nuclei."

BANKER, G., and Cottman, C.W. (1971) Arch. Biochem. 142:565-573. "Characteristics of different amino acids as protein precursors in mouse brain: advantages of certain carboxylic-labelled amino acids."

BANKS, S.P., and Johnson, T.C. (1972) Brain Research 41:155-169. "Maturation - dependent events related to DNA-dependent RNA synthesis in intact mouse brain nuclei."

BANKS, S.P., and Johnson, T.C. (1973) Brain research 62:201-212. "The differential effects of ions on RNA synthesis in nuclei and nucleoli isolated from neonatal and adult mouse brain."

BARONDES, S.H. (1964) Journal of Neurochemistry 11:663-9. "Studies with an RNA polymerase from brain."

BARONDES, S.H. (1969) in Cellular Dynamics of the Neuron Vol. 8. Ed. Barondes, S.H. Academic Press, N.Y. "Two sites of synthesis of macromolecules in neurons."

BARONDES, S.H., and Cohen, H.D. (1968) Proc. natn. Acad. Sci. U.S.A. 61:923-929. "Arousal and the conversion of 'short-term' to 'long-term' memory."

BATESON, P.P.G. (1964) J. Comp. Physiol. Psychol. 58:407-411. "Relation between conspicuousness of stimuli and their effectiveness in the imprinting situation."

BATESON, P.P.G. (1966) Biological Review 41:177-220. "The characteristics and context of imprinting."

BATESON, P.P.G. (1970) pp 553-564 in Short-term Changes in Neural Activity and Behaviour. Ed. G. Horn and A. Hinde, C.U.P. "Are they really the products of learning."

BATESON, P.P.G. (1971) In: The Ontogeny of Vertebrate Behaviour. Ed: Moltz. H. Pub: Academic Press, New York. "Imprinting."

BATESON, P.P.G. (1972) Proc. Int. Ornithological Conference, 1970, pp 303-315. "The formation of social attachments in young birds."

BATESON, P.P.G. (1974) Nature (Lond.) 248:805-807. "Atmospheric pressure during incubation and post-hatch behaviour in chicks."

BATESON, P.P.G., and Reese, E.P. (1969) Animal Behaviour 17:692-699. "The reinforcing properties of conspicuous stimuli in the imprinting situation."

BATESON, P.P.G., and Seaburne-May, G. (1973) Animal Behaviour 21:720-725. "Effects of prior exposure to light on chicks' behaviour in the imprinting situation."

BATESON, P.P.G., and Wainwright, A.A.P. (1972) Behaviour XLII:279-290. "The effects of prior exposure to light on the imprinting process in domestic chicks."

BATESON, P.P.G., Horn G. and Rose, S.P.R. (1969) Nature (Lond.) 223:534-535. "Effects of an imprinting procedure on regional incorporation of tritiated lysine into protein of chick brain."

BATESON, P.P.G., Horn G. and Rose, S.P.R. (1972) Brain Res. 39:449-465. "Effects of early visual experience on regional incorporation of precursors into RNA and protein in the chick brain."

BEATO, M., Homoki, J., Doenecke, D. and Sekeris, C.E. (1970) Experientia 26:1074-1076. "Effects of ions and cortisol on RNA synthesis in lysed rat liver nuclei."

BONDY, S.C., and Morelos, B.S. (1971) Exp. Neurol. 31:200-206, "Stimulus deprivation and cerebral blood flow."

BONDY, S.C., and Roberts, S. (1969) Biochem. J., 115:341-349. "Developmental and regional variations in ribonucleic acid synthesis on cerebral chromatin."

BONDY, S.C., and Waelsch, H. (1965) Journal of Neurochemistry 12:751-756.

BONDY, S.C., Lehman, R.A.W., and Purdy, J.L. (1974) Nature (Lond.) 248:440-441. "Visual attention affects brain blood flow."

BURDMAN, J.A. (1970) J. Neurochem. 17:1555-1562. "Incorporation in vivo of radioactive leucine into neuronal and glial nuclear proteins of rat brain."

BURTON, K. (1956) Biochemical Journal 62:315-323. "A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid."

CHAMBON, P., Ramuz, M., and Doly, J. (1965) Biochem. Biophys. Res. Comm. 21:156-161. "Relation between soluble DNA-dependent RNA polymerase and 'aggregate' RNA polymerase."

CHERKIN, A. (1969) Proc. natn. Acad. Sci. U.S.A. 63:1094-1101. "Kinetics of memory consoldiation: role of amnesic treatment parameters."

CHERKIN, A. (1970) Nature (Lond.) 227:1153. "Eye-to-eye transfer of an early response modification in chicks."

CHURCH, R.B., and McCarthy, B.J. (1970) Biochim. biophys.Acta. 199:103-114. "Unstable nuclear RNA synthesis following estrogen stimulation."

CHURCH, R.M. (1964) Psychol. Bull., 62:122-131. "Systematic effect of random error in the yoked control design."

COHEN, H.D., and Barondes, S.H. (1968) Nature 218:271-273. "Effects of acetoxycycloheximide on learning and memory of a dark/light discrimination."

COLEMAN, M.S., Pfingst, B., Wilson, J.E., and Glassman, E. (1971) Brain Res. 26:349-360. "Brain function and macromolecules: VIII Uridine incorporation into brain polysomes of hypophysectomised rats and ovarietomised mice during avoidance conditioning."

COLEMAN, M.S., Wilson, J.E., and Glassman, E. (1972) Nature (Lond.) 229:54-55. "Incorporation of uridine into polysomes of mouse brain during extinction."

CRAGG, B.G. (1969a) Brain Res. 13:53-67. "The effects of vision and dark-rearing on the size and density of synapses in the lateral geniculate nucleus measured by electron microscopy."

CRAGG, B.G. (1969b) Brain Res. 15:79-96. "Structural changes in naive retinal synapses detectable within minutes of first exposure to daylight."

CUÉNOD, M., Boesch, J., Marko, P., Perisic, M., Sandri, C., and Schonbach, J. (1972) Intern. J. Neuroscience 4:77-87. "Contributions of axoplasmic transport to synaptic structures and functions."

DAVIES, P., and Griffiths, K. (1973) Biochem. J. 136:611-622. "Stimulation of ribonucleic acid polymerase activity in vitro by prostatic steroid-protein receptor complexes."

DIMOND, S.J. (1970) In: Social Behaviour in Birds and Mammals. Ed: J. H. Crook. Pub: Academic Press, London. "Visual experience and early social behaviour in chicks."

DRAVID, A.R., and Duffy, T.E. (1969) Brain Research 16:516-519. "Studies on in vitro RNA synthesis in rat brain nuclei: biphasic stimulation of RNA polymerase with ammonium sulphate."

DUNN, A., Giuditta, A., and Pagliuca, N. (1971) J. Neurochem. 18:2093-2100. "The effect of electroconvulsive shock on protein synthesis in mouse brain."

DUTTON, G.R. and Mahler, H.R. (1968) Journal of Neurochemistry 15:765-780. "In vitro RNA synthesis by intact rat brain nuclei."

EHRET, C.F., and Trucco, E. (1967) J. Theoret. Biol. 15:240-267. "Molecular models for the circadian clock. I. Chronon concept."

EMSON, P.C., Walker, R.J. and Kerkut, G.A. (1971) Comp. Biochem. Physiol. 40B:223-239. "Chemical changes in a molluscan ganglion associated with learning."

FABRICIUS, E. (1951) Acta. Zool. Fenn. 68:1-178. "Zur Ethologie junger Anatiden."

FABRICIUS, E. and Boyd, H. (1954) Wildfowl Trust Ann. Report, 1952-1953; pp 84-89. "Experiments on the following reactions of ducklings."

FIFKOVÁ, E. (1970a) J. Neurobiol. 1:285-294. "The effects of monocular deprivation on the synaptic contacts of the visual cortex."

FIFKOVÁ, E. (1970b) J. Comp. Neurol. 140:431-438. "The effect of unilateral deprivation on visual centres in the rat."

FLEXNER, J.B. and Flexner, L.B. (1967) Proc. natl. Acad. Sci. U.S.A. 67:1651-1654. "Restoration of expression of memory loss after treatment with puromycin."

FLEXNER, J.B. and Flexner, L.B. (1970) Yale J. Biol. Med. 42:235-240. "Further observations on the restoration of memory lost after treatment with puromycin."

FORE, H. and Morton, R.A. (1952) Biochem. J. 51:600 "Manganese in rabbit tissues."

GOLDSTEIN, A. (1973) Nature (Lond.) 242:60-62. Comments on the "Isolation, identification and synthesis of a specific-behaviour-inducing brain peptide."

GRAVES, H.B. and Siegel, P.B. (1974) Anim. Behav. 22:242-248. "Approach responses of Gallus Domesticus chicks: genetic stock, time of day and developmental age effects."

HALJAMÄE, H. and Lange, P.W. (1972) Brain Res. 38:131-142. "Calcium content and conformation changes of S-100 protein in the hippocampus during training."

HAMBLEY, J., and Rose, S.P.R. (1974) Brain Res. (in Press). "Adenosine 3',5'-monophosphate in chick brain following exposure to an imprinting stimulus."

HAMBLEY, J., Haywood, J., Rose, S.P.R. and Bateson, P.P.G. (1974) Brain Res. (in Press). "^{14}C-lysine pool and incorporation in the chick brain after early visual experience."

HAMILTON, T.H., Widnell, C.C., and Tata, J.R. (1968) J. Biol. Chem. 243:408-417. "Synthesis of RNA during early estrogen action."

HAYWOOD, J., Hambley, J. and Rose, S.P.R. (1973) Proc. 4th Meeting of Int. Soc. Neurochem., Tokyo, 1973. "Changes in acetylcholinesterase activity in chick brain following exposure to an imprinting stimulus."

HAYWOOD, J., Hambley, J. and Rose, S.P.R. (1974a) Brain Res. (in Press). "Effects of exposure to an imprinting stimulus on the activity of enzymes involved in acetylcholine metabolism in chick brain."

HAYWOOD, J., Hambley, J., Rose, S.P.R. and Bateson, P.P.G. (1974b) Trans. Biochem. Soc. Vol 2 pp 241-243. "Effects of early visual experience on ^{14}C-lysine incorporation into the chick brain.

HAYWOOD, J., Rose, S.P.R. and Bateson, P.P.G. (1970) Nature (Lond.) 228:373-374. "Effects of an imprinting procedure on RNA polymerase activity in the chick brain."

HAYWOOD, J., Rose, S.P.R. and Bateson, P.P.G. (1974c) Brain Res. (in Press). "Changes in chick brain RNA polymerase following exposure to an imprinting procedure."

HEBB, D.O. (1949) Pub. Wiley, New York. The Organisation of Behaviour.

HERBERT, M., and Sluckin, W. (1969) Anim. Behav. 17:213-216. "Acquisition of colour preferences by chicks at different temperatures."

HERRNSTEIN, R.J. (1966) In: Operant Behaviour: Areas of Research and Application, pp33-51. Ed. W. K. Honig, New York, Appleton-Century-Crofts. "Superstition: a corollary of the principles of operant conditioning."

HESS, E.H. (1959) Science 130:133-141. "Imprinting".

HORN, G. (1967) Nature (Lond.) 215:707-711. "Neuronal mechanisms of habituation."

HORN, G., Rose, S.P.R. and Bateson, P.P.G. (1973) Brain Research 56:227-237. "Monocular imprinting and regional incorporation of tritiated uracil into the brains of intact and 'split-brain' chicks."

HORRIDGE, G.A. (1962) Proc. R. Soc. (B) 157:33-52. "Learning of leg position of the ventral nerve cord in headless insects."

HUNGEN, K. von. (1971) Nature (Lond.) 229:114-115. "Competitive hybridization with brain RNA fails to confirm new RNA induced by learning."

HYDÉN, H., and Egyházi, E. (1962) Proc. natl. Acad. Sci. U.S.A. 48:1366-1373. "Nuclear RNA changes of nerve cells during a learning experiment in rats."

HYDÉN, H., and Egyházi, E. (1963) Proc. natl. Acad. Sci. U.S.A. 49:618-624. "Glial RNA changes during a learning experiment with rats."

HYDÉN, H., and Egyházi, E. (1964) Proc. natl. Acad. Sci. U.S.A. 52:1030-1035. "Changes in RNA content and base composition in cortical neurones of rats in a learning experiment involving transfer of handedness."

HYDÉN, H. and Lange, P.W. (1965) Proc. natl. Acad. Sci. U.S.A. 53:946-952. "A differentiation in RNA response in neurons early and late during learning."

HYDÉN, H. and Lange, P.W. (1970) Proc. natl. Acad. Sci. U.S.A. 67:1959-1966. "S-100 brain protein: correlation with behaviour."

HYDÉN, H. and Lange, P.W. (1972) Proc. natl. Acad. Sci. U.S.A. 69:1980-1984. "Protein changes in different brain areas as a function of intermittent training."

HYMER, W.C. and Kuff, E.L. (1964) J. Histochem. Cytochem. 12:359- . "Isolation of nuclei from mammalian tissues through the use of Triton X-100."

JACOB, M., Stevenin, J., Jund, R., Judes, C. and Mandel, P. (1966) J. Neurochem. 13:619-628. "Rapidly-labelled RNA in brain."

JAKOUBEK, B., Semiginovsky, B., Kraus, M. and Erdossova, R. (1970) Life Sci. 1169-1179. "The alterations of protein metabolism of the brain cortex induced by anticipation stress and ACTH."

JAKOUBEK, B., Buresova, M., Hajek, I., Etrychova, A., Pavlika and Dedicova, A. (1972) Brain Research 43:417-428. "Effect of ACTH on the synthesis of rapidly-labelled RNA in the nervous system of mice."

JAMES, H. (1960) Canad. J. Psychol. 14:13-20. "Imprinting with visual flicker: evidence for a critical period."

JAYNES, J. (1956) J. Comp. Physiol. Psychol. 40:201-206. "Imprinting: the interaction of learned and innate behaviour. I. Development and generalisation."

JOHNSON, E.M., Maeno, H. and Greengard, P. (1971) J. Biol. Chem. 246:7731-7739. "Phosphorylation of endogenous protein of rat brain by cyclic adenosine 3', 5' - monophosphate-dependent protein kinase."

KAHAN, B., Krigman, M.R., Wilson, J.E. and Glassman, E. (1970) Proc. natn. Acad. Sci. U.S.A. 65:300-303. "Brain function and macromolecules. VI. Autoradiographic analysis of the effects of a brief training experience on the incorporation of uridine into mouse brain."

KATO, T. and Kurokawa, M. (1967) J. Cell. Biol. 32:649- . "Isolation of cell nuclei from the mammalian cerebral cortex and their assortment on a morphological basis."

KATO, T. and Kurokawa, M. (1970) Biochem J. 116: 599-609. "Studies on ribonucleic acid and homopolyribonucleotide formation in neuronal, glial and liver nuclei."

KATZ, J.J. and Halstead, W.C. (1950) Comp. Psychol. Monog. 20 :1-38. "Protein organisation and mental function."

KERKUT, G.A., Emson, P. and Walker, R.J. (1973) In: Macromolecules and Behaviour. Ed: G. B. Ansell and P. B. Bradley. Pub: Macmillan. pp 241-257. "Learning in lower animals."

KERKUT, G.A., Oliver, G., Rick, J.T. and Walker, R.J. (1970) Nature (Lond.) 227:722-723. "Biochemical changes during learning in an insect ganglia."

LANGAN, T.A. (1969) J. Biol. Chem. 244:5763-5765. "Action of adenosine, 3',5'-monophosphate-dependent histone kinase in vivo."

LANGENDORF, H., Siebert, G., Lorenz, I., Hannover, R. and Beyer, R. (1961) Biochem. Z. 335:273-284. "Calcium distribution in nuclei and cytoplasm of rat liver."

LASHLEY, K.S. (1950) Symp. Soc. exp. Biol. 4:454-482. "In search of the engram."

LESLIE, I. (1955) In: The Nucleic Acids, Vol.II. Pub: Academic Press, New York.

LINDELL, T.J., Weinberg, F., Morris, P.W. Roeder, R.G. and Rutter, W.J. (1970) Science 170:447-448. "Specific inhibition of nuclear RNA polymerase II by α-amanitin."

LORENZ, K. (1935) J. Orn. 83:137-213. "Der Kumpan in der Umwelt des Vogels."

LØVTRUP-REIN, H. and McEwen, B.S. (1966) J. Cell. Biol. 30:405-415. "Isolation and fractionation of rat brain nuclei."

LOWRY, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193:265-275. "Protein measurement with Folin phenol reagent."

MACHLUS, B. and Gaito, J. (1968) Psychon. Sci. 10:253-4. "Detection of RNA species unique to a behavioural task."

MACHLUS, B. and Gaito, J. (1969) Nature (Lond.) 222:573-574. "Successive competition hybridisation to detect RNA species in a shock-avoidance task."

MACHLUS, B. J., Wilson, J.E. and Glassman, E. (1974a) (In preparation.) "Brain phosphoproteins: I. The effect of short experiences on the incorporation of radioactive phosphate into nuclear proteins of rat brain."

MACHLUS, B.J., Wilson J.E. and Glassman, E. (1974b) (In preparation." "Brain phosphoproteins: II. The effect of various behaviours and reminding experiences on the incorporation of radioactive phosphate."

MANDEL, P., Dravid, A.R. and Pete, N. (1967) Journal of Neurochemistry 14:301-306. "Poly-C synthetase activity in the particulate fraction of rat brain nuclei."

MARGOLIS, F.L. (1969) J. Neurochem. 16:447-456. "DNA and DNA-polymerase activity in chicken brain regions during ontogeny."

MARR, D. (1970) Proc. R. Soc. (B) 176:161-234. "A theory for cerebral neocortex."

MAUL, G.G. and Hamilton, T.H. (1967) Proceedings of the National Academy of Sciences, U.S.A. 57:1371-1378. "The intranuclear localisation of two DNA-dependent RNA polymerase activities."

MEIER, R.E. and Cuénod, M. (1970) Experientia (Basel). 26:682. "A visual function of the supraoptic decussation in the pigeon."

MERRITT, J.H. and Sulkowski, T.S. (1970) J. Neurochem. 17:1327-1328. "Rhythmicity of RNA polymerase activity and RNA levels in nuclei from rat cerebral cortex."

MERTELSMANN, R. (1969) European Journal of Biochemistry 9:311-318. "Purification and some properties of a soluble DNA-dependent RNA polymerase from nuclei of human placenta."

METZGER, H.P., Cuénod, M., Grynbaum, A. and Waelsch, H. (1967) J. Neurochem. 14:183-187. "The effect of unilateral visual stimulation on synthesis of cortical proteins in each hemisphere of the split brain monkey."

MOLTZ, H. (1963) Psychol. Rev. 70:123-138. "Imprinting:an epigenetic approach."

MOLTZ, H. and Stettner, L. J. (1962) J. Comp. Physiol. Psychol. 55:626-632. "Interocular mediation of the following response after patterned light deprivation."

McEWEN, B.S. and Zigmond, R.E. (1972) In: Methods in Neurochemistry Vol. I. Ed: Marks and Rodnight. Pub: Plenum Press, New York.

McEWEN, B.S., Plapinger, L., Wallach, G. and Magnus, C. (1972) Journal of Neurochemistry 19:1159-1170. "Properties ofcell nuclei isolated from various regions of rat brain: divergent characteristics of cerebellar cell nuclei."

NURNBERGER, J.I. (1958) In: Biology of Neuroglia. Ed: W. F. Windle. Pub: Thomas, Ill., U.S.A. pp 193-202 "Direct enumeration of cells of the brain."

OJA, S.S. (1967) Annales Academiae Scientiarum Fennicae (A) 131:3-83. "Studies on protein metabolism in developing rat brain."

OJA, S.S. (1973) International Journal of Neuroscience 5:31-33. "Comments on the measurement of protein synthesis in the brain."

OKAZAKI, K., Shull, K.H. and Farber, E. (1968) J. Biol. Chem. 243:4661- . "Effects of ethionine on adenosine triphosphate levels and ionic composition of liver cell nuclei."

OLIVER, R.W.O., Taberner, P.V., Rick, J.T. and Kerkut, G. A. (1971) Comp. Biochem. Physiol. 38B:529-535. "Changes in GABA level, GAD and ChE activity in CNS of an insect during learning."

PEARSON, R. (1972) The Avian Brain. Pub: Academic Press, London.

POGO, A.O., Littau, V.C., Allfrey, V.G. and Mirsky, A.E. (1967) Proc. natn. Acad. Sci. U.S.A. 57:743-750 "Modification of ribonucleic acid synthesis in nuclei isolated from normal and regenerating liver: some effects of salt and specific divalent cations."

REES, H.D., Brogan, L.L., Entingh, D.J., Dunn, A.J., Shinkman, P.G., Damstra-Entingh, T., Wilson, J.E. and Glassman, E. (1974) Brain Res. 68:143-156. "Effect of sensory stimulation on the uptake and incorporation of radioactive lysine into protein of mouse brain and liver."

RICHARDSON, K. and Rose, S.P.R. (1971) Nature (Lond.) 233:182-184. "A diurnal rhythmicity in incorporation of lysine into rat brain regions."

RICHARDSON, K. and Rose, S.P.R. (1972) Brain Res. 44:299-303. "Changes in ^{3}H-lysine incorporation following first exposure to light."

ROEDER, R.G. and Rutter, W.J. (1969) Nature (Lond.) 224:234-237 "Multiple forms of DNA-dependent RNA polymerase in enkaryotic organisms."

ROEDER, R.G. and Rutter, W.J. (1970) Proceedings of the National Academy of Sciences, U.S.A. 65:675-682. "Specific nucleolar and nucleoplasmic RNA polymerases."

ROSE, S.P.R. (1967) Nature (Lond.) 215:253-255. "Changes in visual cortex of first exposure of rats to light."

ROSE, S.P.R. and Sinha, A.K. (1974) "Incorporation of amino acids into proteins in neuronal and neuropil fractions of rat cerebral cortex: presence of a rapidly labelling neuronal fraction."

ROSE, S.P.R., Bateson, P.P.G., Horn, G. and Horn, A.L.D. (1970) Nature (Lond.) 225:650-651. "Effects of an imprinting procedure on regional incorporation of tritiated uracil into chick brain RNA."

ROSE, S.P.R., Sinha, A.K. and Broomhead, S. (1973) J. Neurochem. 21:539-546. "Precursor incorporation into cortical protein during first exposure of rats to light: cellular localisation of effects."

ROSENZWEIG, M.R., Love, W. and Bennett, E.L. (1968) Physiol. Behav. 3:819-825. "Effects of a few hours a day of enriched experience on brain chemistry and brain weights."

ROSENZWEIG, M.R., Bennett, E.L., Diamond, M.C., Su-Yu, W.W., Slagle, R.W. and Saffran, E. (1969) Brain Res. 14:427-445. "Influences of environmental complexity and visual stimulation of development of occipital cortex in rat."

SALZEN, E.A. (1967) In: Development and Evolution of Behaviour, Ed: L. R Aronson. Pub: Freeman, New York. "Imprinting and environmental learning."

SCHAEFER, H.H. and Hess, E.H. (1959) Z. Tierpsychol. 16:161-172. "Colour preferences in imprinted objects."

SCHEIN, M.W. (1963) Z. Tierpsychol. 20:462-467. "On the irreversibility of imprinting."

SCHNEIDER, W.C. (1945) Journal of Biological Chemistry. 161:293-303. "Phosphorus compounds in animal tissue. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid."

SCHOTMAN, P., Gipon, L. and Gispen, W.H. (1974) Brain Res. 70:377-380. "Conversion of [4,5-^{3}H] leucine into $^{3}H_2O$ and tritiated metabolites in rat brain tissue: comparison of a peripheral and intracranial route of administration."

SCHUTZ, F. (1965) Psychol. Forsch. 28:439-463. "Homosexualitat und Pragung."

SHASHOUA, V.E. (1968) Nature (Lond.) 217:238-240. "RNA changes in goldfish during learning."

SHASHOUA, V.E. (1970) Proc. natl. Acad. Sci. U.S.A. 65 : 160-167 "RNA metabolsim in goldfish brain during acquisition of new behavioural patterns."

SIEGEL, P.B. and Siegel, H.S. (1964) Anim. Behav. 12:270-271. "Rearing methods and subsequent sexual behaviour of male chickens."

SIGGINS, G.R., Hoffer, B.J. and Bloom, F.E. (1969) Science 165:1018-1020. "Cyclic adenosine monophosphate: possible mediator for norepinephrine effects on cerebellar Purkinje cells."

SLAGEL, D.E. and Akers, R.D. (1972) Brain Research 44:245-260. "Isolation, fractionation and RNA polymerase activity in a sample enriched with oligodendroglia nuclei."

SLUCKIN, W. (1972) Imprinting and Early Learning Pub: 2nd. Edn. Methuen, London.

SMITH, F.V. (1969) Attachment of the Young. Pub: Oliver and Boyd, Edinburgh.

SMITH, F.V. and Hoyes, P.A. (1961) Anim. Behav. 9:159-166. "Properties of the visual stimuli for the approach response in the domestic chick."

SOKOLOV, E.N. (1960) In: The Central Nervous System and Behaviour. Ed: M.A.B. Brazier. Pub: Josiah Macy Jnr. Foundation, New York. "Neuronal models and the orienting reflex."

SPALDING, D.A. (1873) Macmillan's Magazine. 27:282-293. "Instinct with original observations on young animals."

SQUIRE, L.R., Smith, G.A. and Barondes, S.H. (1973) Nature 242:201-202. "Cycloheximide affects memory within minutes after the onset of training."

STEINER, D.F. and King, J. (1966) Biochim. Biophys. Acta. 119:510-516. "Insulin-stimulated ribonucleic acid synthesis and RNA polymerase activity in alloxam-diabetic rat liver."

STEWART, W.W. (1972) Nature (Lond.) 238:202-209. "Comments on the chemistry of scotophobin."

TATA, J.R. (1968) Nature (Lond.) :331-337. "Hormonal regulation of growth and protein synthesis."

THIERS, R.A. and Vallee, B.L. (1957) J. Biol. Chem. 226:911- "Distribution of metals in subcellular fractions of rat liver."

THOENEN, H. (1974) Life Sciences 14:223-235. "Trans-synaptic enzyme induction."

THOMPSON, R.J. (1973) J. Neurochem. 21:19-40. "Studies on RNA synthesis in two populations of nuclei from the mammalian cerebral cortex."

THORPE, W.H. (1956) Learning and Instinct in Animals. Pub: Methuen, London.

THORPE, W.H. (1961) In: W. H. Thorpe and O. L. Zangwill, Current Problems in Animal Behaviour. Pub: Cambridge University Press, Cambridge. pp 194-224. "Sensitive periods in the learning of animals and men: a study of imprinting with special reference to the induction of cyclic behaviour."

TIPLADY, B. (1972) Brain Research 43:215-225. "Brain protein metabolism and environmental stimulation, effects of forced exercise."

TIPLADY, B. (1974) Life Sci. (In press) "Brain protein metabolism and forced exercise: involvement of a rapidly-labelled protein fraction."

TRES, L.L., Kierszenbaum, A.L. and Tandler, C.J. (1972) J. Cell. Biol. 53:483-493. "Inorganic cations in the cell nucleus. Selective accumulation during meiotic prophase in mouse testis."

UNGAR, G., Galvan, L. and Clark, R.H. (1968) Nature (Lond) 217:1259-1261. "Chemical transfer of learned fear."

UNGAR, G., Desiderio, D.M. and Parr, W. (1972) Nature (Lond.) 238:198-202. "Isolation, identification and synthesis of a specific-behaviour-inducing brain peptide."

UPHOUSE, L.L., MacInnes, J.W. and Schlesinger, K. (1974) Behaviour Genetics 4:29-81. "Role of RNA and protein in memory storage: a review."

WATTS, M.E. and Mark, R.F. (1970) Proc. R. Soc. Lond. B 178:455-464. "Drug inhibition of memory formation in chickens. II. Short-term memory."

WIDNELL, C.C. and Tata, J.R. (1966) Biochemica et Biophysica Acta 123:478-492. "Studies on the stimulation by ammonium sulphate of the DNA-dependent RNA polymerase of isolated rat liver nuclei."

ZAMENHOF, S., Gravel, L., van Marthens, E. and Stillinger, R.A. (1972) J. Neurochem. 19:61-68. "Quantitative determination of DNA in preserved brains and brain sections."

ZAMENHOF, S., van Marthens, E. and Bursztyn, H. (1971) Proc. Int. Symp. on Hormones in Development. Appleton-Century-Croft, New York. "The effect of hormones on DNA synthesis and cell number in the developing chick and rat brain."

ZEMP, J.W., Wilson, J.E. and Glassman, E. (1967) Proc. Natn. Acad. Sci. U.S.A. 58:1120-1125. "Brain function and macromolecules. II. Site of increased labelling of RNA in brains of mice during a short-term training experience."

ZEMP, J.W., Wilson, J.E., Schlesinger, K., Boggan, W.O. and Glassman, E. (1966). Proc. natn. Acad. Sci. U.S.A. 55:1423-1431. "Brain function and macromolecules: I. Incorporation of uridine into RNA of mouse brain during short-term training experience."

ZILVERSMIT, D.B., Entenman, C. and Fishler, M. (1943) J. Gen. Physiol. 26:325-331. "On the calculation of 'turnover time' and 'turnover rate' from experiments involving the use of labelling agents."